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The journal is fulfilling this aim by publishing full-length original research papers, rapid papers, invited reviews, At the Cutting Edge essays, and book reviews.

The scope encompasses all subjects related to biochemical and molecular aspects of endocrine research and cell regulation. These include (1) mechanisms of action of extracellular signals (hormones, neurotransmitters, etc.), (2) interaction of these factors with receptors, (3) generation, action and role of intracellular signals such as cyclic nucleotides and calcium, (4) hormone-regulated gene expression, (5) structure and physicochemical properties of hormones, hormone receptors and other hormone-binding components, (6) synthesis, secretion, metabolism and inactivation of hormones, neurotransmitters, etc., (7) hormonal control of differentiation, (8) related control mechanisms in non-mammalian systems, (9) methodological and theoretical aspects related to hormonal control processes, (10) clinical studies as far as they throw new light on basic research in this field, (11) control of intermediary metabolism at the cellular level, (12) ultrastructural aspects related to hormone secretion and action.

Editors

C.W. Bardin
The Population Council
Center for Biomedical Research
1230 York Avenue
New York, NY 10021 (U.S.A.)
Tel. + 1–212–570.8717
Fax + 1–212–570.7678

B.A. Cooke (Managing Editor)
Department of Biochemistry
Royal Free Hospital School of Medicine
Rowland Hill Street
London NW3 2PF (U.K.)
Tel. + 44–(0)71–794.0500 ext. 4202/4989
Fax + 44–(0)71–794.5029

P. Corvol
Pathologie Vasculaire et
Endocrinologie Rénale
Institut National de la Santé
et de la Recherche Médicale
3 rue d' Ulm
75005 Paris (France)
Tel. + 33–(0)1–44.27.1674
Fax + 33–(0)1–44.27.1691

J.E. Dumont
Institute of Interdisciplinary Research
Faculty of Medicine
Free University of Brussels
Campus Erasme, Bldg. C
808 Route de Lennik
B-1070 Brussels (Belgium)
Tel. + 32–(0)2–5264.134
Fax + 32–(0)2–5264.226

J.W. Funder (Review Editor)
Baker Medical Research Institute
P.O. Box 348
Prahran, Vic. 3181 (Australia)
Tel. + 61–(0)3–522.4333
Fax + 61–(0)3–510.4368

G.N. Gill
Department of Medicine, M-050
Division of Endocrinology/Metabolism
University of California, San Diego
La Jolla, CA 92093-0650 (U.S.A.)
Tel. + 1–619–534.4310

D.K. Granner
Vanderbilt University
Department of Molecular Physiology
and Biophysics
Nashville, TN 37232-0615 (U.S.A.)
Tel. + 1–615–322.7004
Fax + 1–615–322.7236

J.A. Grootegoed
Department of Biochemistry II
Medical Faculty
Erasmus University Rotterdam
P.O. Box 1738
3000 DR Rotterdam (The Netherlands)
Tel. + 31–(0)10–40.87.345
Fax + 31–(0)10–43.66.832

G. Schultz
Freie Universität Berlin
Institut für Pharmakologie
Thielallee 69–73
D-1000 Berlin 33 (F.R.G.)
Tel. + 49–(0)30–838.6360
Fax + 49–(0)30–831.5954

E.R. Simpson
(Associate Managing Editor)
Cecil H. and Ida Green Center for
Reproductive Biology Sciences
The University of Texas
Southwestern Medical Center at Dallas
5323 Harry Hines Boulevard
Dallas, TX 75235-9051 (U.S.A.)
Tel. + 1–214–688.3260
Fax + 1–214–688.8683

J.R. Tata
National Institute for Medical Research
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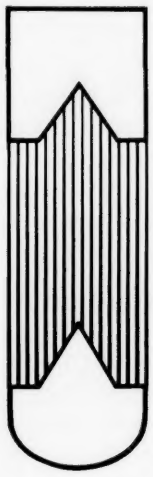

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At the Cutting Edge

Thyroid hormone regulation of rat pituitary tumor cell growth: a new role for apotransferrin as an autocrine thyromedin

David A. Sirbasku, Rajbabu Pakala, Hidetaka Sato * and John E. Eby

Department of Biochemistry and Molecular Biology, University of Texas Medical School, Houston, TX 77225, U.S.A.

Key words: Pituitary; Growth; Thyroid hormone; Transferrin; Apotransferrin; Cell–cell adhesion

This cutting edge review has two goals. First, we will describe some of the major advances leading to our present understanding of thyroid hormone-dependent pituitary tumor cell growth. The discussion particularly will emphasize the theme of hormonal/autocrine control of cell proliferation. Second, we will review recent studies in serum-free defined culture which have led to the identification of apotransferrin as an essential regulator of thyroid hormone-dependent pituitary cell growth.

Milestones in thyroid hormone growth regulation

Jacob Furth (b. 1896–d. 1979) made a vital contribution to our understanding of pituitary cell physiology. Along with several colleagues, he established a collection of rodent tumors which retained many of the functional properties of the adenohypophysis (Furth, 1953, 1969; Furth et al., 1956; Yokoro et al., 1961). Most notably, several tumors secreted growth hormone and/or prolactin. One of these, the Wistar-Furth rat derived MtT · W5, was used by Tashjian et al. (1968) to establish the now famous GH_1 and GH_3 cell lines in culture. Later, the GH_4C_1 (Tashjian et al., 1970) and the GC (Bancroft and Tashjian, 1971) subclones were obtained. All have been used in the study of thyroid hormone mechanism of action.

Samuels et al. (1973) were first to report thyroid hormone-dependent growth of the GH_1 cells in culture. This was a milestone in our understanding of thyroid hormone mechanism of action. Later, they showed that growth was mediated by the binding of triiodothyronine (T_3) or L-thyroxine (T_4) to specific nuclear receptors (Samuels and Tsai, 1973; Samuels et al., 1974).

Hayashi and Sato (1976) provided another milestone by achieving GH_3 cell growth in a serum-free defined medium containing a mixture of nutrients and hormones. Between 1976 and 1984, Gordon Sato and colleagues demonstrated that many different cell types grew in serum-free defined medium (Bottenstein et al., 1979; Barnes and Sato, 1980; Sato et al., 1982; Barnes et al., 1984). These studies led to the description of many previously unidentified physiological responses/regulators (Barnes et al., 1987).

At present, there is little doubt that nuclear thyroid hormone receptors mediate the proliferative response of rat pituitary tumor cells in culture (see review Oppenheimer et al., 1987). Nonetheless, the question remained of whether this inter-

Address for correspondence: David A. Sirbasku, Ph.D., Department of Biochemistry and Molecular Biology, The University of Texas Medical School, 6431 Fannin Street, Houston, TX 77225, U.S.A. Tel. (713) 792-5600; Fax (713) 794-4150.

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* On leave from The Department of Internal Medicine, Research Institute for Cancer and Chest Diseases, Tohoku University, 4-1 Seiryomachi, Sendai 980, Japan.

action alone was sufficient to regulate the cascade of events culminating in cell division. The studies of Samuels et al. (1973) were done in serum supplemented medium. Conservatively estimated, this meant that at least 5000 components other than T_3/T_4 were present; sorting through this number for putative new regulatory molecules appeared to be a formidable task.

Fortunately, the reports of Hayashi and Sato (1976) and Hayashi et al. (1978) provided a method to resolve this problem. They grew GH_3 cells in a defined medium supplemented with T_3, diferric transferrin, partially purified insulin-like growth factor I (IGF-I), parathyroid hormone and thyrotropin releasing hormone. Since all five were components of serum, defined culture methods offered a promising means of reducing 5000 possibilities to a much smaller number. However, despite the great success with GH_3 cells, there were two findings of note. Deletion of thyroid hormones from defined media had only modest consequences, compared with removal of some of the other components. Secondly, the magnitude of the T_3 growth response in defined medium was markedly lower than in serum containing culture.

At about this time, another milestone was reached which inspired an entirely different approach to growth regulation. DeLarco and Todaro (1978) found that tumor cells secreted polypeptide mitogens. Later, these were classified as 'autocrine growth factors' (Sporn and Todaro, 1990), and within a few years became the center of the tumor biology universe (see review by Goustin et al., 1986). Very quickly, other investigators applied this concept to endocrine tumors; Dickson et al. (1986), for example, reported the estrogen-induction of autocrine mitogens for human breast cancer cells and we examined pituitary cell conditioned medium and tumor extracts for possible 'autocrine' activities (Danielpour et al., 1984).

Others also were seeking autocrine factors for rat pituitary tumor cells. Fagin et al. (1987) identified IGF-I as a secretory product of GH_3 cells. Because these cells expressed type I (IGF-I) receptors (Rosenfeld et al., 1985), an 'autocrine growth loop' was suggested. However, despite the presence of the basic elements of such a loop, it was not possible to confirm IGF-I regulation of pituitary cell proliferation in serum-free defined medium (Riss et al., 1989; Sirbasku et al., 1991b). In fact, other data suggested that IGF-I instead modulated growth hormone production by negative feedback (Yamashita and Melmed, 1986).

Hinkle and Kinsella (1986) provided the next piece of the jig-saw puzzle. Using serum containing cultures, they reported thyroid hormone induction of potent autocrine (secreted) factor(s) which completely replaced the T_3 requirement of the GH_4C_1 cells. A subsequent report by Miller et al. (1987) described similar results with GC cells. Not only did these findings provide a timely autocrine mechanism for thyroid hormone induced growth, but they also implied that this new activity might act for T_3/T_4 in the myriad of metabolic processes, including regulation of energy metabolism and control of osmotic pumps, known to be influenced by these hormones (Oppenheimer et al., 1987).

Initial serum-free study of pituitary cell growth

Our initial plan was to grow rat pituitary tumor cells in T_3 supplemented serum-free defined culture and prepare conditioned medium for characterization of this thyroid hormone substituting activity, a strategy beset by many problems (Stewart and Sirbasku, 1987, 1988a, b; Riss et al., 1989).

First, $< 10\%$ of the GH_3 and GH_4C_1 cells survived passage from serum containing culture into defined medium. This problem persisted despite variations in plating density, medium composition and methods of preparation of inoculum cells. Second, the surviving cells required supraphysiological concentrations of T_3 (i.e. 10 nM) and rapidly progressed to autonomy. Indeed, these conditions permitted isolation of hormone-autonomous variants (Riss et al., 1989). Third, the effects of polypeptide growth factors were different from those reported (Hayashi and Sato, 1976; Hayashi et al., 1978; Bottenstein et al., 1979). Deletion of insulin and/or IGF-I had no effect on growth or survival. Members of the epidermal growth factor and the fibroblast growth factor-like families were inhibitory. Transforming growth factor β and platelet derived growth factors were without effect. In summary, none of the five major functional families of non-lymphoid growth fac-

tors supported pituitary cell proliferation in defined medium with or without T_3. By inference, the T_3 substituting activity of Hinkle and Kinsella (1986) probably was not a member of a major growth factor family. In addition, we were unable to identify a T_3 substituting activity from serum-free cultures of GH_4C_1 cells.

When the problem was reconsidered, a different interpretation of the data of Hinkle and Kinsella (1986) appeared possible. Conceivably, one of more component(s) of the serum may have been converted to an active agent during incubation of the cells with T_3. In view of results to be described below, we believe that 'autocrine' apotransferrin generated by recycling of diferric transferrin may be related to the T_3 substituting activity. Finally, thyroid hormone-responsive GH_1 cells failed to grow in any formulation of defined medium including those with T_3 and insulin. In contrast, addition of low concentrations of thyroid hormone depleted serum restored cell survival and thyroid hormone-dependent growth. These data were convincing evidence that serum contained a thyroid hormone mediating activity designated 'thyromedin' (Stewart and Sirbasku, 1987, 1988a, b).

Isolation and characterization of thyromedins

We purified thyromedins from horse serum (Sirbasku et al., 1991a, b) and human plasma (Sato et al., 1991). Bioassays in two related serum-free media were used to monitor the isolations. GH_1 cell growth in PCM-10 containing Ham's F12 and Dulbecco's modified Eagle's medium (1 : 1) supplemented with 2.2 g/l sodium bicarbonate, 15 mM Hepes (pH 7.2), 10 μg/ml diferric transferrin, 50 μM ethanolamine, 10 μg/ml insulin, 10 ng/ml selenous acid, 500 μg/ml serum albumin and 0.1 nM T_3 was compared to proliferation in the same medium without T_3 (PCM-0). Chromatographic fractions supporting growth in PCM-10, but not in PCM-0, were purified to electrophoretic and HPLC homogeneity.

From horse serum, > 600 mg/l of thyromedin was obtained as seven separate forms (Sirbasku et al., 1991a). Human plasma yielded > 500 mg/l represented by three forms (Sato et al., 1991). The quantities obtained, as well as the striking polymorphism, raised the expectation that thyromedin was a previously unrecognized activity of a known serum protein. In summary, all forms/variants or horse and human thyromedins proved to be transferrin by molecular weight estimation, Fe^{3+} binding capacity, Western immunoblotting versus authentic samples, amino acid composition analysis and N^{α}-amino acid sequencing.

Properties of thyromedin biological activity

The results presented in this and the next section summarize three recent reports (Sato et al., 1991; Sirbasku et al., 1991a, b). Initially, growth assays were done in medium prepared with standard Gibco F12-DME containing ferric nitrate and ferrous sulfate salts. The total iron content of PCM-10 was 65 ng/ml as measured by atomic absorption spectroscopy. If all of the iron was Fe^{3+}, this cocnentration was enough to saturate 46 μg/ml of apotransferrin assuming two Fe^{3+}/molecule.

The ED_{50} of molecular weight 80,000 horse thyromedin IIIa (53% Fe^{3+} saturated) in 'iron salts containing' PCM-10 was 15–20 μg/ml (285 nM); $\geq$ 25 μg/ml was needed for maximal growth (Fig. 1*A*). In 7 days, cell numbers in PCM-10 exceeded those in PCM-0 by 3–4 cell population doublings (CPD) or 800–1600%. It was notable that even at 100 μg/ml, horse thyromedin IIIa failed to achieve ED_{50} in PCM-0.

The same experiment with molecular weight 80,000 human thyromedin gave comparable results (Fig. 1*B*). Form Ia, which was 9% Fe^{3+} saturated, had an ED_{50} of 12 μg/ml. GH_1 cell numbers in PCM-10 after 7 days exceeded PCM-0 by 5–6 CPD or up to 6400%. Even 100 μg/ml of human Ia did not promote ED_{50} growth in PCM-0.

Although thyromedins from both sources clearly were effective mediators of T_3 induced growth, a number of questions arose. First, the ED_{50} values, as well as the concentrations required to saturate growth, were near μM. This was disconcerting because physiological regulators are expected to be active at lower concentrations. Second, diferric transferrin was present in PCM-10 at 10 μg/ml, yet the medium did not support growth.

Thyromedin activity and apotransferrin

Addressing these problems led to the first identification of a serum-derived mediator of hormone-dependent cell growth since our search began (Sirbasku, 1978). The following is a summary of the evidence supporting the conclusion that thyromedins are apotransferrin:

Iron content of thyromedins and activity. When the seven forms of horse thyromedin were analyzed for iron content versus biological activity, an important pattern emerged. Those showing the lowest ED_{50} concentrations (i.e. highest specific activity) were $\leq 50\%$ iron saturated whereas others approaching 80–90% saturation were inactive, or nearly so, when assayed in standard 'iron salts containing' media. The three human thyromedins showed an identical pattern. Thus, lower iron content clearly correlated with higher biological activity.

Iron saturation and thyromedin activity. All seven horse thyromedins and the three human forms were iron saturated with ferric ammonium citrate as described (Ward and Kaplan, 1987). Assays in standard 'iron salts containing' PCM-10 showed no biological activity with any thyromedin at concentrations of up to 100 μg/ml. Fig. 1*C* shows an example assay of iron saturated human thyromedin Ia.

Iron depletion and thyromedin activity. Next, iron was depleted from the thyromedins and from the tissue culture medium before bioassay. Water was purified to reduce iron content (Sirbasku et al., 1991a). Iron salts free F12-DME was obtained from Gibco. The final concentration of iron in 'iron salts reduced' PCM-10 was < 10 ng/ml. All horse and human thyromedins were dialyzed against 0.1 M sodium citrate, pH 4.0, for 48 h at 4°C to reduce the iron content to $< 4\%$ saturation (Roop and Putnam, 1967). Assays in 'iron salts reduced' medium gave ED_{50} values of 80–180 ng/ml. Fig. 1*D* shows an example assay of citrate-treated human thyromedin Ia in 'iron salts reduced' media. All thyromedins were 100- to 2000-fold activated under iron reduced conditions compared to those with iron present. These low

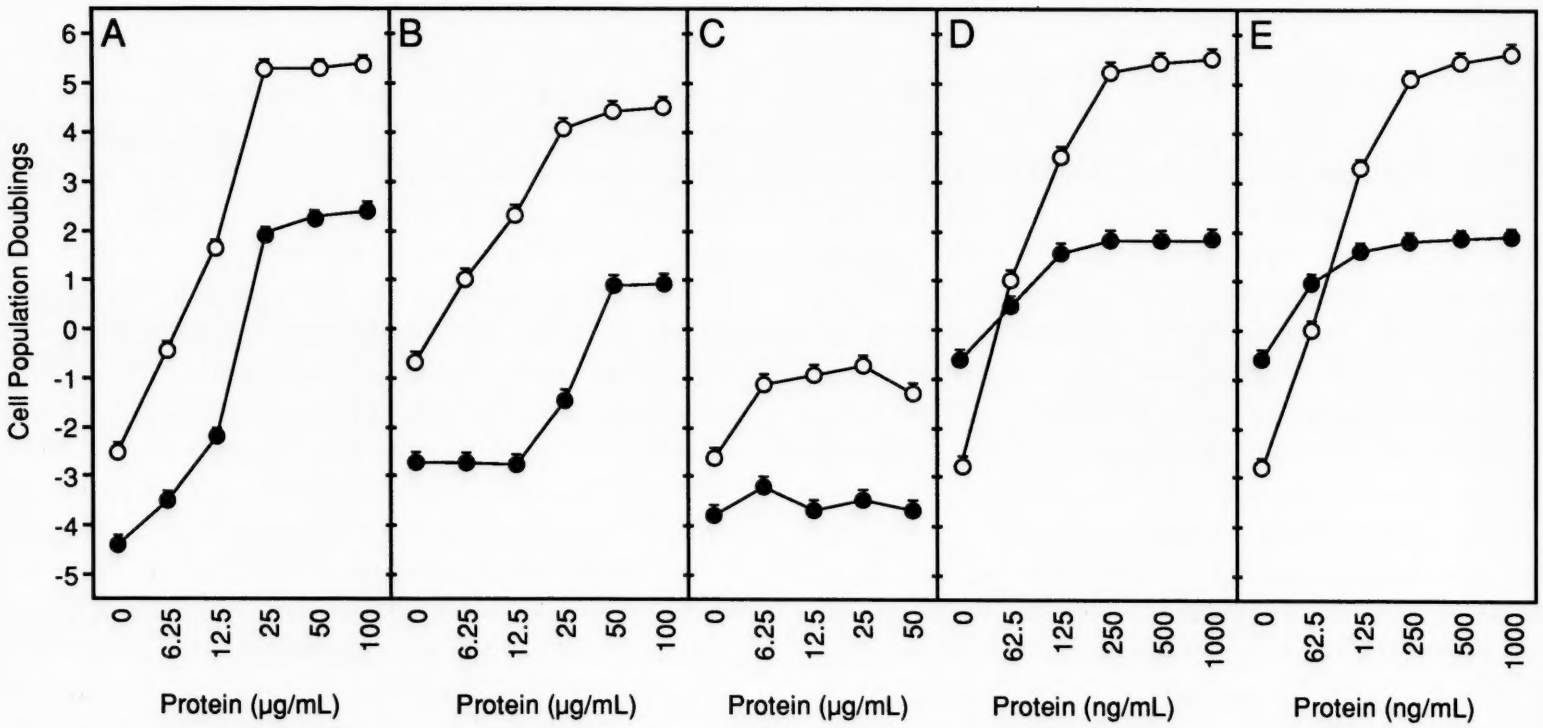

Fig. 1. Dose–response growth of GH_1 cells in PCM-10 (open circles) and PCM-0 (closed circles) with the following preparations/conditions: (*A*) horse thyromedin IIIa (Sirbasku et al., 1991a) in 'iron salts containing' media; (*B*) human thyromedin Ia (Sato et al., 1991) in 'iron salts containing' media; (*C*) Fe^{3+} saturated human Ia in 'iron salts containing' media; (*D*) iron depleted (citrate dialyzed) human Ia in 'iron salts reduced' media; and (*E*) Fe^{3+} saturated human Ia in 'iron salts reduced' media. Assays were over 7 days ($\pm$ SD, bars).

nM concentrations at which activity is seen are thus consistent with those at which other physiological regulators operate.

Deferoxamine activation of thyromedin activity. Another test was made of the effect of the iron concentration of the medium on thyromedin activity. Deferoxamine mesylate (Sigma) is a low molecular weight chelator which binds one Fe^{3+}/molecule with much higher affinity than apotransferrin (Peter, 1985). When deferoxamine mesylate was added to 'iron salts containing' medium to chelate all available Fe^{3+}, the effect on the biological activity of thyromedins was striking. Horse thyromedin IIIa, which showed an ED_{50} of 20 μg/ml before deferoxamine addition (Fig. 1*A*), increased 100-fold in activity (to an ED_{50} of 200 ng/ml) after preincubation of the medium with the chelator. This observation had two important implications. First, it explained why our initial preparations were less active than expected. With the favorable conditions of tissue culture, the Fe^{3+} of 'iron salts containing' medium associated with apotransferrin to form inactive diferric transferrin. Second, this finding supported the conclusion that apotransferrin was the active mediator of thyroid hormone-dependent cell growth.

Iron salts addition and thyromedin activity. As shown in Fig. 1*D*, citrate-treated human thyromedin Ia gave an ED_{50} of 100 ng/ml in 'iron salts reduced' PCM-10. In another test of the effect of iron on activity, increasing concentrations of ferric ammonium citrate were added to 'iron salts reduced' medium in identical experiments using horse IIIa and human Ia thyromedins. The activities of both citrate treated preparations were inhibited progressively by the iron salt proving that medium composition regulated biological potency.

Assay of apotransferrins and diferric transferrins from several species. Commercially prepared horse, human, rat, mouse, dog, guinea pig and rabbit transferrins were either Fe^{3+} saturated as described (Ward and Kaplan, 1987) or depleted of iron by exhaustive citrate dialysis (Roop and Putnam, 1967). Assay of the iron depleted proteins in 'iron salts reduced' PCM-10 resulted in ED_{50} values of 80–350 ng/ml and little or no growth in PCM-0. The results paralleled the assay of human thyromedin Ia under the same conditions (Fig. 1*D*) and affirmed that apotransferrins from several species were effective. The iron recharged transferrins were assayed in 'iron salts containing' PCM-10 and PCM-0 exactly as described in Fig. 1*C*; no activity was found at concentrations of up to 200 μg/ml. These results not only proved that diferric transferrins were not thyromedins but also explained why PCM-10 containing 10 μg/ml human or horse diferric transferrin did not support growth.

What form of apotransferrin is present in tissue culture medium? Although these studies demonstrated that ferric ion binding to apotransferrin resulted in the loss of thyromedin activity, they did not address the possibility of association of other metals with the apoprotein. This is an important consideration because apotransferrin binds non-ferrous ions including zinc, copper and trace metals (see review by Harris, 1989). In 'iron salts reduced' medium, 70–80% of apotransferrin is associated with metal ions. Because Zn^{2+} is present at 1.5 μM in PCM-10 and binds to the apoprotein with a K_a of $10^{7.8}$, our present studies are focused on the possible role of Zn^{2+}-apotransferrin in hormone-dependent cell growth.

'Autocrine loop' formation of apotransferrin

Despite the purification of apotransferrin/thyromedin from plasma, we questioned whether this source was important physiologically. Most mammalian cells produce apotransferrin via recycling of the complex of diferric transferrin and the molecular weight 180,000 dimeric cell surface receptor (Ciechanover et al., 1983; May and Cuatracasas, 1985). A schematic summary of this process is shown in Fig. 2. At extracellular neutral pH, diferric transferrin binds with nM affinity to receptors located in clathrin coated pits. The pits are internalized to form coated endosomes followed by acidification of the vesicles to pH 5–6 via action of a H^+-ATPase and dissociation of the Fe^{3+} from transferrin. At acidic pH, apotransferrin remains tightly associated with the receptors. The iron depleted endosomes recycle to the cell surface where fusion exposes the apotransferrin/receptor complex to the extracellular environment. With most cell types studied, apotransferrin rapidly dissociates from the receptor at neutral pH.

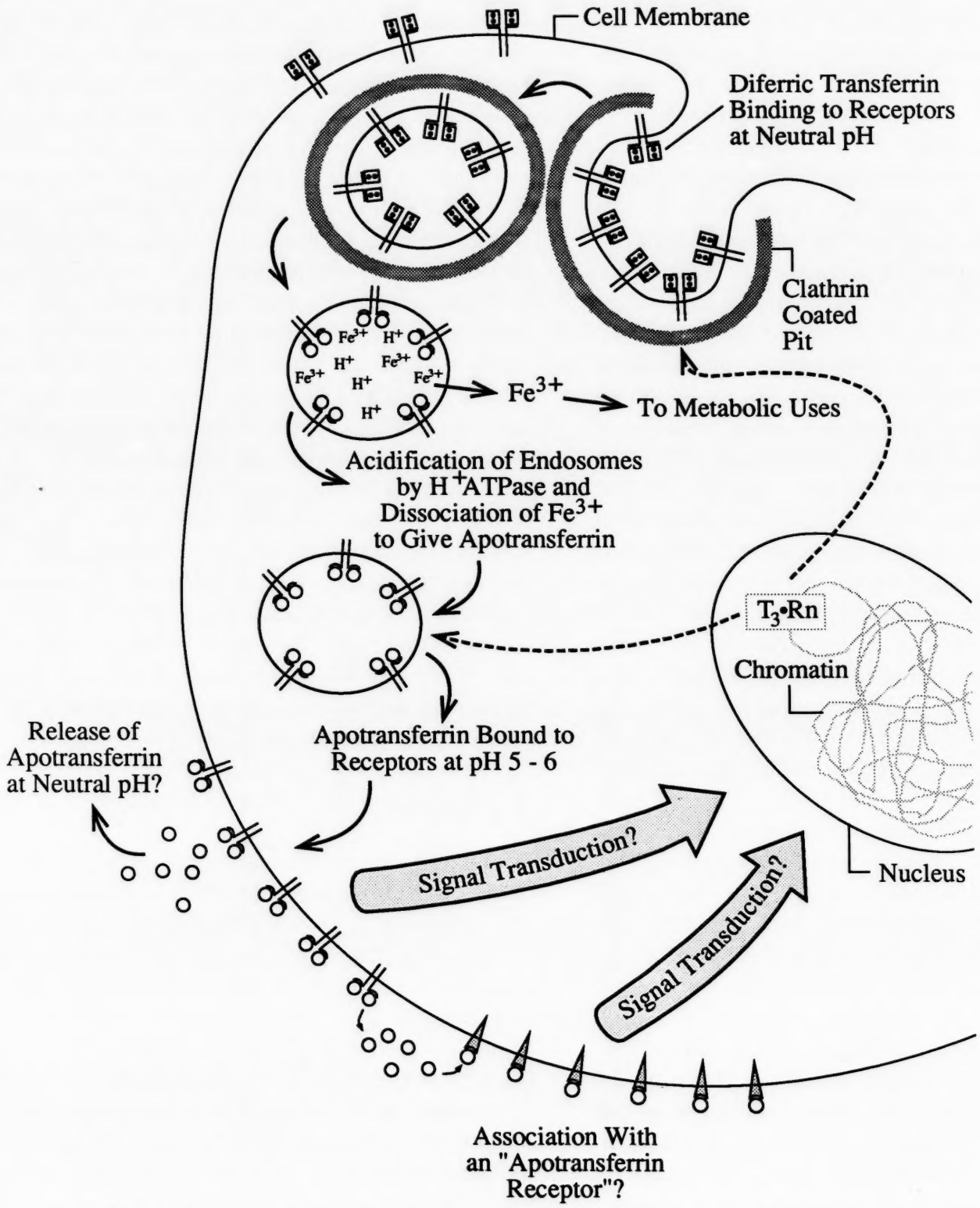

Fig. 2. Schematic summary of the cycling pathway of diferric transferrin internalization to produce apotransferrin.

Although we expected the same cycle in GH_1 cells, experimental confirmation was sought. Iron saturated human thyromedin Ia effectively promoted GH_1 growth in 'iron salts reduced' medium (Fig. 1*E*). It should be noted that this same preparation was inactive in 'iron salts containing' medium (Fig. 1*C*). The results confirmed that apotransferrin generation by recycling of diferric transferrin represented an 'autocrine growth loop' for pituitary tumor cells.

These observations raise important questions. First, do thyroid hormones regulate the rate of diferric transferrin recycling and thereby control 'autocrine' apotransferrin production (Fig. 2)?

Second, does apotransferrin control grow.h via binding to the molecular weight 180,000 dimeric diferric transferrin receptor or instead function via an apotransferrin specific receptor, as proposed in Fig. 2? In the former case, the usually rapid dissociation of apotransferrin from the diferric transferrin receptor at neutral pH may be altered.

Cell–cell adhesion and apotransferrin

Along with growth regulation, thyromedin/apotransferrin showed another interesting new property. GH_1 cells plated into PCM-0 or PCM-10 alone floated above the dish surface without interactions (Fig. 3*A*). When thyromedin was added to PCM-0, the cells aggregated and attached as clusters to the dish surface (Fig. 3*B*). Neurite-like projects were apparent. Addition of thyromedin to PCM-10 not only stimulated growth, but also the formation of large aggregates attached more firmly to the dish surface (Fig. 3*C*). The morphology in PCM-10 plus thyromedin was identical to that of cultures grown in serum (Fig. 3*D*).

The cell–cell interactions observed in the presence of apotransferrin implied a cell adhesion molecule (CAM) activity. Although most CAMs are in the immunoglobulin superfamily or are lectins, the structure of apotransferrin suggested a new possibility. Transferrin is a bilobular molecule with similar 'N' and 'C' lobes sharing 35–45% amino acid sequence homology (Baker et al., 1987). Each lobe has a '1' and '2' domain positioned so that the same amino acid sequence of the 'N' lobe is facing in the opposite direction as that of the 'C' lobe. This arrangement might recognize equivalent receptors on adjacent cells thereby initiating cell–cell contact.

In addition to the possibility of cell–cell bridges formed by apotransferrin, another adhesion mechanism was conceivable. Ramsdell (1990) identified

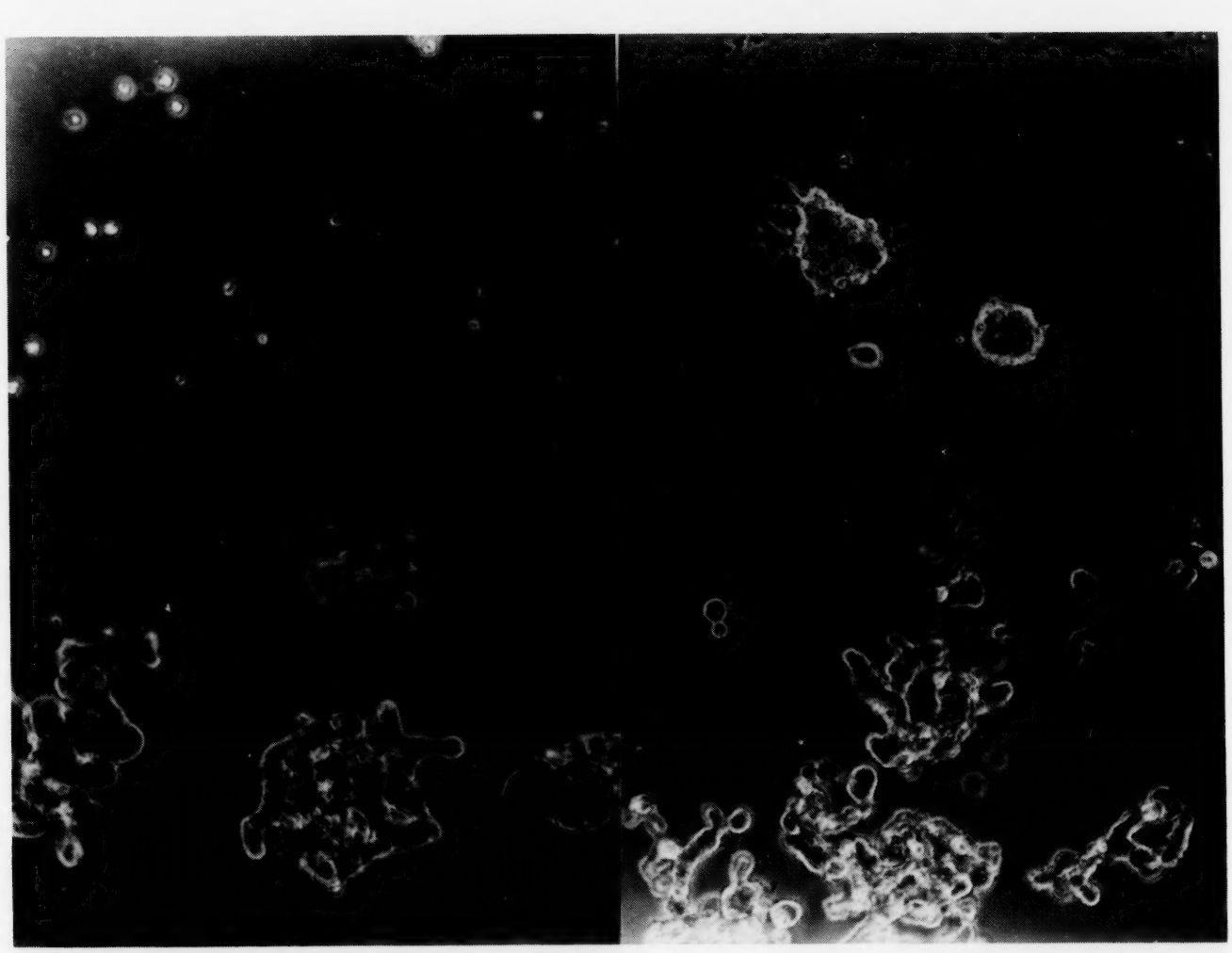

Fig. 3. Photomicrographs of GH_1 cells growing/inoculated into serum-free defined PCM-10 (or PCM-0) only (*A*), PCM-0 plus horse thyromedin (*B*), PCM-10 plus thyromedin (*C*), and PCM-10 plus 5% horse serum (*D*). ×100.

neural cell adhesion molecules (N-CAMs) on GH_4C_1 cell surfaces and studied their role in cell–cell contact. Apotransferrin might induce N-CAM expression and thereby promote cell–cell interactions. Whichever mechanism(s) proves important, the adhesion observations are especially exciting because they raise the possibility that cell–cell interactions might be important in thyroid hormone induced cell proliferation.

Summary

In the 40 years of transferrin research, no previous role for apotransferrin has been recognized other than to serve as a plasma carrier for dietary and storage iron. Our studies have revealed a new 'autocrine' growth role for this molecule as well as a possible new cell–cell bridge/CAM function. Certainly, these observations have opened many new areas of investigation both with regard to thyroid hormone action and the function of apotransferrin. In addition, there is now accessible the broader question of tissues other than pituitary which might utilize apotransferrin to regulate responsiveness to thyroid hormones.

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At the Cutting Edge

Insulin-like growth factor I (IGF-I): a molecular basis for endocrine versus local action?

D. LeRoith and C.T. Roberts, Jr.

Molecular and Cellular Physiology Section, Diabetes Branch, National Institutes of Diabetes and Digestive and Kidney Diseases, National Institute of Health, Bethesda, MD 20892, U.S.A.

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Key words: Insulin-like growth factor-I; Endocrine; Paracrine

Hormones have traditionally been considered substances elaborated by specialized tissues (endocrine glands) and subsequently transported to distant target cells via the circulation. Growth factors have, in contrast, been defined as substances that are synthesized and secreted by a wide variety of cells and tissue types, which then act through paracrine or autocrine mechanisms after diffusion through the extracellular fluids (Sporn and Roberts, 1988). Although the separation between endocrine and paracrine/autocrine systems is valid in certain instances, there are numerous aspects of each that overlap, making such a distinction somewhat artificial; for example, insulin, a hormone, can also affect growth in certain tissues. The major elements in each case, including ligands, receptors and post-receptor mechanisms are often the same. To further complicate matters, recent evidence suggests that to promote growth and differentiation certain growth factors do not need to be secreted to interact with their targets. In some instances, for example, partially processed growth factors are retained in the plasma membrane of the cell of origin, presumably to interact more efficiently with receptors on adjacent target cells (Brachmann et al., 1989), a 'juxtacrine' mode of action. Alternatively, growth factors may remain within their cell of origin and regulate cellular function entirely by acting intracellularly (Neufeld et al., 1988; Fleming et al., 1989; Heldin and Westermark, 1989), an 'intracrine' mode of action.

The insulin-like growth factors, IGF-I and IGF-II, are mitogenic polypeptides which are structurally related to insulin (Rinderknecht and Humbel, 1978; Blundell et al., 1983; Froesch and Zapf, 1985). Originally, these factors were thought to be produced exclusively by the liver, in the case of IGF-I (somatomedin C) in response to growth hormone (GH) (Salmon and Daughaday, 1957). Following GH administration, there is an increase in circulating levels of these IGFs which reach their target organs, primarily growing bone, via the circulation. This 'somatomedin hypothesis' has now been revised, since IGFs are synthesized in many non-hepatic tissues in both fetal and adult animals (D'Ercole et al., 1980, 1984; Han et al., 1987; Murphy et al., 1987a; Roberts et al., 1987a). Furthermore, the biological actions of IGFs are not limited to tissue growth, since they can also induce the terminal differentiation of many cell types, raising the question as to whether they are hormones, local factors or both (Florini et al., 1986; Clautres et al., 1987; Lin et al., 1988).

The biological actions of the IGFs are initiated by their binding to specific cell surface receptors. These receptors include the closely related insulin

Address for correspondence: D. LeRoith, M.D., Ph.D., Diabetes Branch, NIDDK, NIH, Building 10, Room 8S243, 9000 Rockville Pike, Bethesda, MD 20892, U.S.A.

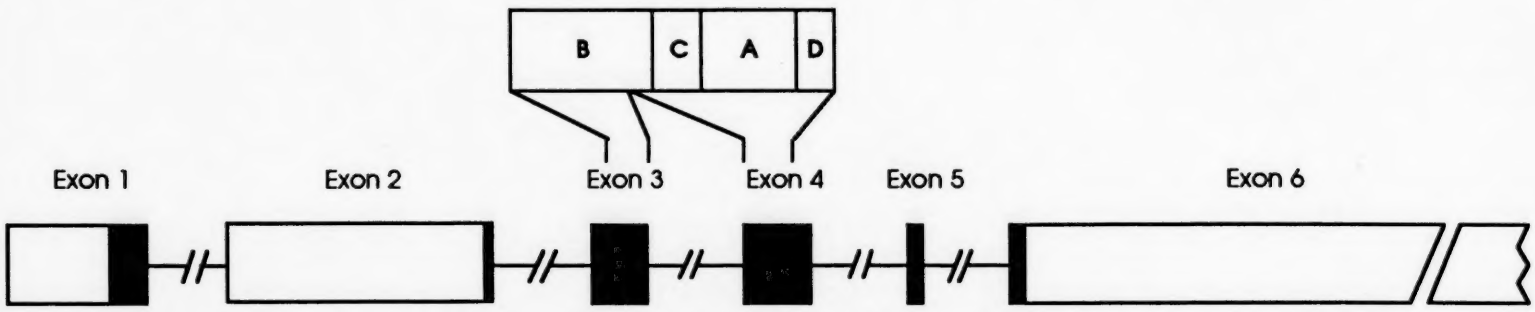

Fig. 1. Structure of the rat IGF-I gene. Translated regions are in black and introns and exon 6 are not to scale.

and IGF-I receptors, which exhibit ligand-induced tyrosine kinase activity (Rechler and Nissley, 1985; Ullrich et al., 1986; Steele-Perkins et al., 1988), and the IGF-II/mannose-6-phosphate receptor, which may act via G protein activation (Nishimoto et al., 1987, 1989). IGF-binding proteins are also an important component of this complex system. These proteins transport IGFs in the circulation and in extracellular fluids, and thus may regulate ligand–receptor interactions either by blocking the interaction of ligand with receptor or, under certain circumstances, by enhancing receptor-mediated functions (Elgin et al., 1987; Clemmons, 1991).

Our understanding of this complex system has been greatly enhanced by the recent characterization of many components of the system at the genetic level. The nucleotide and derived amino acid sequences of a number of the ligands, binding proteins and receptors have been determined, and the structures of a number of the genes characterized. This information has helped in identifying which tissues synthesize the IGFs, their binding proteins and receptors, as well as to identify factors involved in the regulation of the expression of the genes encoding them.

In mammalian species studied, IGF-I is encoded by a single-copy gene and the mature circulating peptide of 70 amino acids contains B and A domains, as originally described for insulin (Rotwein et al., 1986; Shimatsu and Rotwein, 1987). The mature form of IGF-I, however, retains the C peptide and also contains a short carboxy-terminal extension, termed the D domain. Both cDNA and genomic sequences suggested the presence of an unexpected additional carboxyterminal E peptide, not originally found in circulating IGF-I. In the case of human, rat and mouse, two different E peptides are encoded by alternately spliced IGF-I mRNAs (Bell et al., 1986; Rotwein et al., 1986; Roberts et al., 1987a). These E peptides are presumably cleaved off during the processing and maturation of preproIGF-I.

More detailed genetic analysis has increased our understanding of the regulation of IGF-I gene expression in the rat and, in particular, suggested differential regulation of IGF-I in different tissues. The rat IGF-I gene is very large (> 70 kb in length) consisting of at least six exons separated by five introns (Fig. 1) *. Exons 3 and 4 encode the B, C, A and D domains of the mature peptide, as well as 27 amino acids of signal peptide and the first 16 amino acids of the E peptide. Exons 1 and 2 encode the remainder of the signal peptide as well as 5′-untranslated sequences. Exons 5 and 6, on the other hand, encode alternative E peptides, and exon 6 encodes the 3′-untranslated region. Alternate splicing of these various 5′ and 3′ exons results in multiple mRNA transcripts; these may range in size from 0.8–1.2 kb up to 7.5 kb, primarily due to differential polyadenylation site usage in exon 6 (Lund et al., 1989).

Of particular interest is the alternate splicing of exons at the 5′ end of the gene. Two major classes of mRNA transcripts have been identified in hepatic and non-hepatic tissues, each coding for the same mature peptide but containing different 5′-untranslated regions, derived from transcription initiation at different transcription start sites in exons 1 and 2 (Adamo et al., 1991). Exon 2 transcripts are expressed primarily in liver, whereas exon 1 transcripts are the major transcripts found

* The nomenclature used in this article conforms to the consensus developed at the 2nd International Symposium on IGFs in San Francisco, CA, January 12–16, 1991.

in all tissues, including liver. The effect of GH on choice of transcription initiation sites was studied in hypophysectomized rats; a marked decrease in exon 2-derived transcripts (formerly termed class A and B) was seen in liver following hypophysectomy, with a 10-fold increase after repeated GH injections (Lowe et al., 1987). In contrast, the steady-state levels of exon 1 mRNA transcripts in all tissues studied were significantly less affected by these manipulations. Developmental regulation of these transcripts has similarly been investigated; a postnatal increase in exon 1-containing transcripts was found to begin before the GH-dependent increase in serum IGF-I, i.e. prior to postnatal day 20 in the rat. Interestingly, exon 2 transcripts began to increase only between postnatal days 20 and 30, corresponding to the period of the GH-dependent increase in circulating IGF-I and longitudinal growth (Adamo et al., 1988).

Based upon these results, we suggest that exon 2 transcripts regulated primarily by GH perhaps encode the 'endocrine' form of IGF-I. Similarly, exon 1 transcripts may be considered to encode the paracrine/autocrine form of IGF-I since, as noted above, exon 1 mRNA transcripts are expressed in all tissues studied. The production of IGF-I by non-hepatic tissues is regulated by a number of factors other than GH, such as estrogen in ovary, placenta and bone, gonadotropins in ovary and testes, and insulin in the aorta (Murphy et al., 1987b). We and others have shown, in fact, that estrogen and not GH is primarily responsible for regulating exon 1 mRNA transcripts and IGF-I production in ovarian tissue (Hernandez et al., 1989).

The role of the IGF-I E peptides has yet to be determined. We have studied the relative expression of the mRNAs encoding the different E peptides in hepatic and non-hepatic tissues (Lowe et al., 1988). As described above. IGF-I mRNAs can encode two forms of the E peptide, specifically due to the presence or absence of exon 5 sequences. Interestingly, IGF-I mRNAs encoding the Ea form of the E peptide (which lack the exon 5 sequence) are the major transcripts in all tissues, whereas IGF-I mRNAs encoding the Eb form of the E peptide (which contain the exon 5 sequence) while also found in all tissues, are more abundant in liver. In addition, Eb IGF-I mRNA levels are somewhat more sensitive to GH in liver than in other tissues. Thus, the Eb form of the E peptide appears to be more associated with endocrine actions of IGF-I than the predominant Ea form of the E peptide, although this correlation is not as striking as that with the different 5′ variants. Comparison of the amino acid structure of each E peptide has demonstrated the presence of two N-linked glycosylation sites present in the Ea form of the E peptide but not in the Eb form of the E peptide raising the question as to whether glycosylation of the Ea version of the E peptide is involved in the processing or biological action of this prohormone species (Bach et al., 1990).

Another, and potentially more significant, post-translational modification of IGF-I is the N-terminal truncation of the B domain of mature (or pro-) IGF-I (Carlsson-Skwirut et al., 1986; Sara et al., 1986). The cleavage of three N-terminal amino acids (gly-pro-glu; GPE) results in an IGF-I molecule that is more potent than the circulating mature IGF-I molecule, on the basis that their removal decreases the affinity of the truncated form of IGF-I for IGF-binding proteins, thus allowing increased binding to IGF receptors (Carlsson-Skwirut, 1989). To date, tissue as opposed to circulating IGF-I has been found to consist of this N-terminally-cleaved molecule, leading Vicki Sara and her coworkers to propose that this may represent the local/paracrine form of IGF-I, and the circulating full-length molecule the endocrine form (Sara and Hall, 1990).

Given these data it may be appropriate to consider mechanisms whereby differential exon usage may regulate the production of endocrine and autocrine/paracrine forms of IGF-I. As illustrated in Fig. 2, exon 1-containing IGF-I mRNAs encode a 48-amino acid signal peptide, whereas the great majority of exon 2-containing IGF-I mRNAs encode a 32-amino acid signal peptide. While the C-terminal 27 amino acids of each are identical, the N-terminal 21 amino acids of the exon 1-encoded signal peptide and the five N-terminal amino acids of the exon 2-encoded signal peptides diverge. In light of recent studies implicating structural features in the N-terminus of eukaryotic signal peptides as a factor determining the site of cleavage by signal peptidase (Nothwehr and Gordon, 1990), we suggest that the

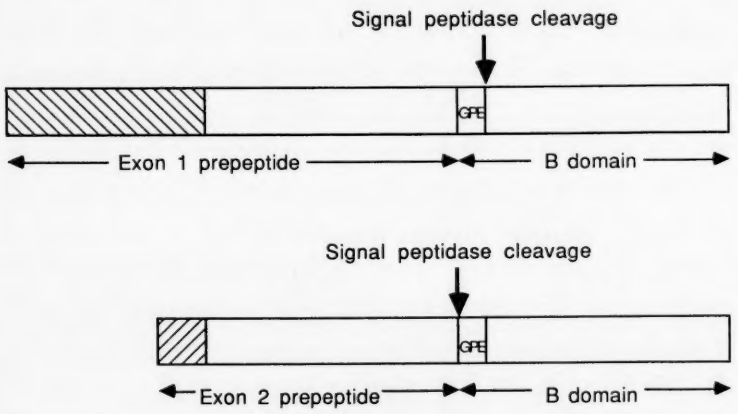

Fig. 2. Schematic representation of the proposed differential processing of the exon 1 and exon 2-encoded versions of preproIGF-I by signal peptidase.

exon 1-specific form of preproIGF-I may be cleaved by signal peptidase to generate a proIGF-I molecule already lacking the N-terminal tripeptide of the B domain. The exon 2-encoded preproIGF-I molecule, on the other hand, would be processed to generate a complete B domain and would be secreted normally into the circulation to effect the endocrine functions of IGF-I. Such a possibility also raises the question of a potential role for the N-terminal peptide itself.

In conclusion, the role of IGFs in normal growth and development, and in controlling the function of numerous differentiated systems, is obviously very complex. The structure of the IGF-I gene allows for multiple levels of regulation at transcriptional, translational and post-translational steps. This review proposes an hypothesis on the possibility of differential regulation of IGF-I gene expression reflecting the different roles of the molecule in endocrine and paracrine systems and suggests a functional correlation between the molecular biology and the physiology of IGF-I.

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At the Cutting Edge

Temporal architecture of in vivo endocrine glandular signaling: obtaining a secretory blueprint by deconvolution analysis

Johannes D. Veldhuis

Interdisciplinary Graduate Biophysics Program, Division of Endocrinology and Metabolism, Department of Internal Medicine, Biodynamics Institute, University of Virginia Health Sciences Center, Charlottesville, VA 22908, U.S.A.

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Key words: Pulsatile; Hormone; Secretory; Episodic

Why study secretion?

Recent molecular and cellular studies of the mechanisms that regulate endocrine glandular secretion have unmasked an array of interesting biochemical and molecular features of hormone release. Such mechanisms involve genomic control, subcellularly compartmentalized hormone synthesis, posttranslational (glyco)protein modification, and appropriate intragranular packaging for exocytosis. Moreover, studies in cell biology have identified discrete messenger pathways activating secretion in individual cells, cell ensembles, and intact endocrine glands.

Despite these advances in our understanding of endocrine secretory mechanics, far less information is available regarding the regulated time-structure of hormone release. The temporal architecture of endocrine glandular signaling is believed to convey important biochemical information to the remote target tissue, and also represents a signature of the time-specified activation of responsive endocrine cells. Consequently, to assemble an overall understanding of endocrine glandular pathophysiology, detailed knowledge of the time domain of hormone synthesis, processing, and release is obligatory. Indeed, recent studies indicate that remarkable changes in distinct aspects of the time course of endocrine glandular activation can be observed in various pathophysiological conditions, such as puberty, healthy aging, the normal menstrual cycle, obesity, diabetes mellitus, acromegaly, thyrotoxicosis, etc. (e.g. Mauras et al., 1987; Hartman et al., 1990; Veldhuis et al., 1990).

The time-structure of endocrine glandular signaling can often be described quantitatively by the frequency, duration, and amplitude of relevant hormone secretory events, and in some cases by the concomitant expression of basal (inter-pulse) hormone secretion (Veldhuis et al., 1987). In addition to this model of punctuated secretory bursts, earlier formulations tended to embrace ultradian (periods of less than 24 h) and circadian (approximately 24-h periodic) rhythms of secretion. However, such ostensibly regular sinusoidal rhythms may arise in part from the dynamics of time-series methods, since by many criteria serial secretory burst intervals are apparently random and not strictly related (Butler et al., 1986; Veldhuis et al., 1989a, b). Accordingly, electrical engineering constructs of regularly repeating cycles often represent an oversimplification of secretory behavior.

Address for correspondence: Johannes D. Veldhuis, Interdisciplinary Graduate Biophysics Program, Division of Endocrinology and Metabolism, Department of Internal Medicine, Biodynamics Institute, University of Virginia Health Sciences Center, Charlottesville, VA 22908, U.S.A., Tel. (804) 924–9697; Fax (804) 982–3626.

Where did classical circadian and ultradian endocrine rhythms go?

Recent concepts of the time-stipulated behavior of endocrine glands have allowed an even more parsimonious construct of secretory pathophysiology. Specifically ultradian and circadian rhythms in agonist concentrations can be envisioned to result from hormone-specific neuroendocrine regulatory mechanisms that control the amplitude and/or frequency of secretory bursts, which are otherwise randomly dispersed over 24 h or shorter intervals (Veldhuis et al., 1990). Thus, for example, the classically identified rhythms in serum

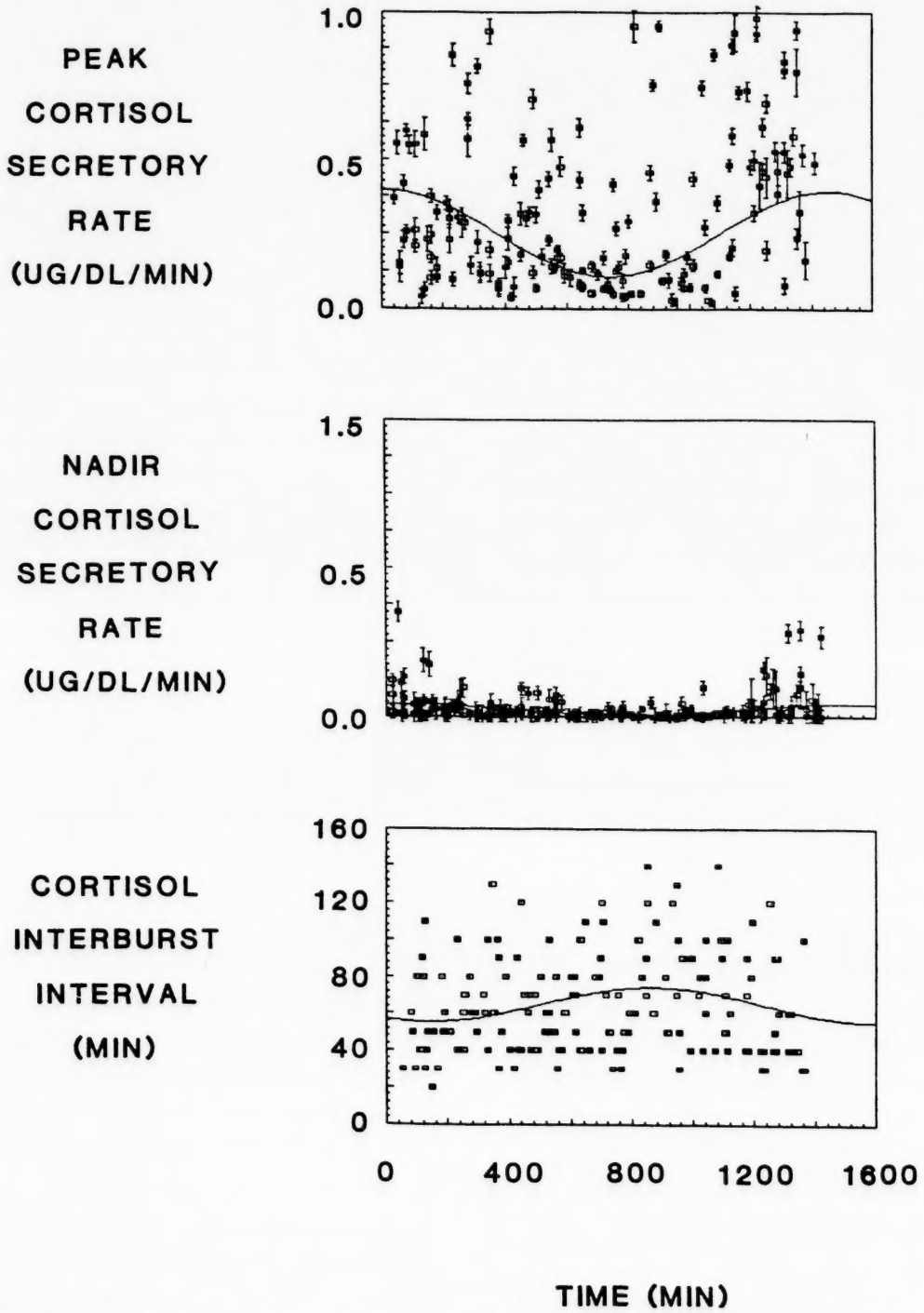


Fig. 1. Circadian rhythms in specific features of cortisol secretory events. Deconvolution analysis was applied to profiles of serial serum cortisol concentrations measured in blood collected at 10-min intervals for 24 h in eight normal men. All peak and nadir values of the individual cortisol secretory pulses were located in time, and the duration (and mid-point in time) of all interburst intervals specified. Both peak cortisol secretory rates (upper panel) and interburst intervals (lower panel) varied significantly over 24 h as assessed statistically by cosinor analysis, but nadir cortisol secretory rates were not distinguishable from zero (middle panel). (J.D. Veldhuis, M.L. Johnson, A. Iranmanesh, G. Lizarralde, unpublished.)

concentrations of anterior pituitary hormone (e.g. ACTH, growth hormone, etc.) and target tissue products (e.g. cortisol) can be represented adequately by a frugal model of randomly distributed hormone secretory bursts, the amplitude and/or frequency of which are subject to regulation over 24 h (circadian rhythm) or a shorter interval (ultradian rhythms): see Fig. 1. In this regulatory system, no variation in a basal or constitutive secretory component is required to account for the full diurnal behavior of many endocrine glands, and the timing of maximal secretion can be closely coupled to relevant environmental and/or endogenous cues.

What is all this talk of 'deconvolution'?

An important technology that has made possible quantitative statements about endocrine glandular signaling in vivo is deconvolution analysis (Veldhuis et al., 1988b). This technique has been reviewed in detail elsewhere (Veldhuis and Johnson, 1990), and comprises a class of numerical methodologies for extracting the time structure of underlying secretion from serial plasma hormone concentrations. The strategy of deconvolution differs from earlier approaches to evaluating serum hormone concentration patterns, in that deconvolution attempts to unmask underlying secretory events by mathematically 'stripping' away the effects of relevant elimination kinetics that otherwise confound the observed hormone concentration profile. Thus, deconvolution consists of 'unraveling, uncoiling, or disentwining' the separated secretion and/or elimination contributions to the observed serum hormone concentrations.

Deconvolution analysis can be carried out in a variety of technically distinct ways, which can be categorized broadly as either secretion waveform-specific or secretion waveform-independent. By waveform-specific, I mean those techniques of deconvolution that require knowledge of or an assumption about the nature or form (time course) of the underlying secretory activity. For example, the underlying secretion waveform might be assumed to be: (a) a square-wave function in which secretion onset and offset are nearly instantaneous; (b) a Gaussian function in which a particular hormone secretory burst is represented as a population of individual molecular secretory rates centered about some instant in time and dispersed with some finite standard deviation; or (c) other waveforms: see Fig. 2. In contrast, waveform-independent models of deconvolution have no necessary assumptions about the time profile of underlying secretory activity; i.e. secretion could occur in bursts of any particular time-shape, occur continuously, or occur as individual sample impulses (Veldhuis and Johnson, 1990).

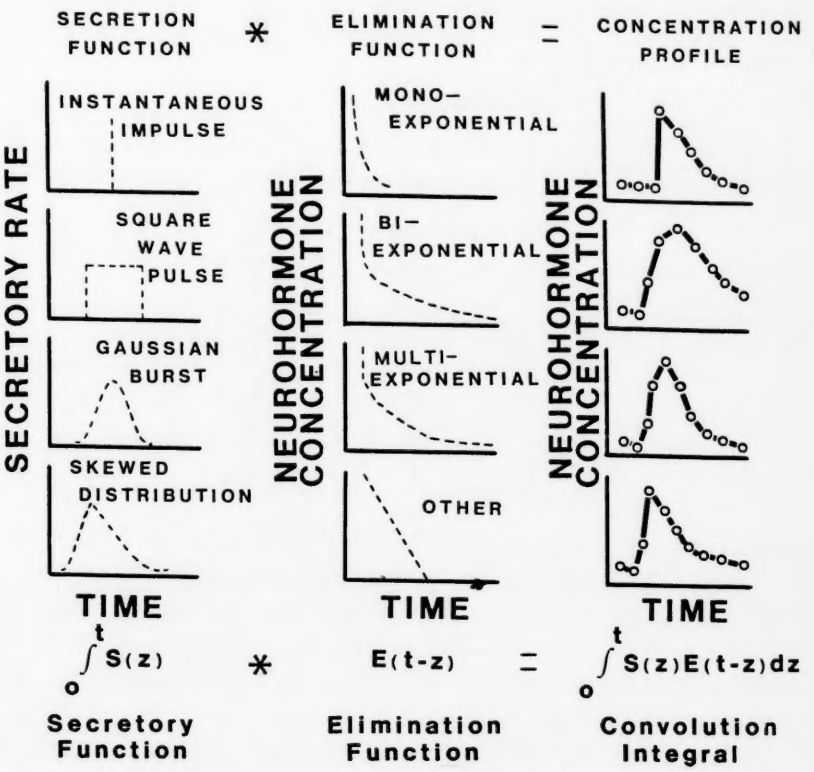
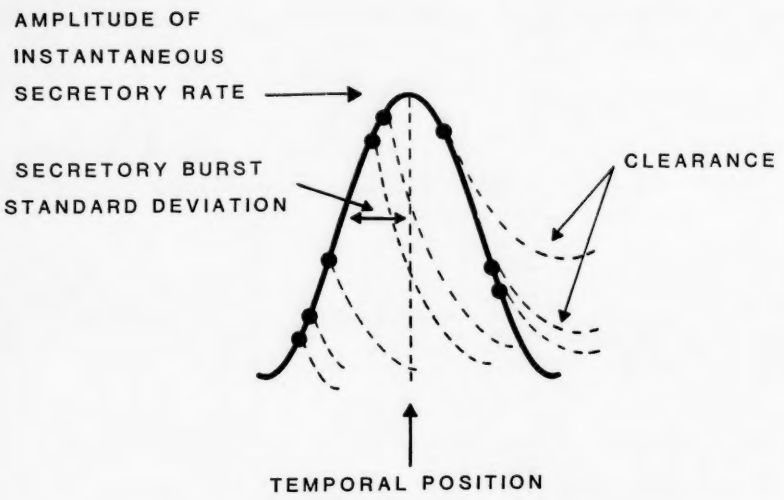


Fig. 2. Schematic illustration of the concept of a convolution integral. A convolution integral is used to specify the simultaneous effects of two functions operating to control the overall observed outcome. For example, the convolution of a secretion function (leftmost panels) and a clearance or elimination function (middle panels) when integrated with respect to time will describe a profile of hormone, metabolite, or substrate concentration over time (rightmost panels). A variety of potential secretion functions could be employed to represent the temporal behavior of hormone release rates in a particular circumstance. For example, a secretion episode may be represented as a nearly instantaneous impulse, a 'square-wave' event, a symmetric Gaussian burst, etc. In addition, the kinetic function to describe the amount of hormone eliminated per unit time could consist of one or more exponential terms, a concentration-dependent function, a partially saturable process, etc. (Adapted with permission from Veldhuis, J.D. and Johnson, M.L. (1990) J. Neuroendocrinology (in press).)

The waveform-specific approach to estimating glandular secretion has as an important strength,



Model of Combined Secretion & Clearance

Fig. 3. Schema of a hypothetical hormone secretory burst. In this model, a secretory *burst* results from the discharge of hormone from a cell or collection of cells within an endocrine ensemble. Numerous molecules of hormones are released, each at its own theoretical secretory rate or velocity (v_i). The *set* of such secretory velocities constitutes a secretory burst or pulse. This set of secretory velocities will have a mean value and variance about the mean. In some cases, the set of secretory velocities in a burst may approach a *Gaussian* distribution. The latter can be used to approximate many other distributions when i is large (i is the number of discharged molecules per burst). Such a Gaussian distribution can be defined fully by its mean, standard deviation, and maximum. All secreted molecules are assumed to be subject to hormone-specific elimination kinetics (metabolic clearance). (Adapted with permission from Veldhuis, J.D., Carlson, M.L. and Johnson, M.L. (1987) Proc. Natl. Acad. Sci. U.S.A. 84, 7686.)

the ability to calculate not only secretion but also metabolic clearance in a hormone- and subject-specific manner (Veldhuis et al., 1987). Thus, a series of observed hormone concentrations can be understood to originate from a finite number of hormone secretory bursts with quantitatively determinable amplitudes, durations, and locations in time. One can then not only estimate these particular features of putative hormone secretory events, but also simultaneously calculate the apparent half-life of hormone disappearance from the sampling compartment: see Fig. 3. This approach to evaluating the temporal architecture of in vivo endocrine glandular secretory activity has revealed a range of alterations in hormone secretory dynamics in various pathophysiological states. For example, there are profound changes in the number, amplitude, and duration of LH secretory events throughout the normal human menstrual cycle, whereas the total daily secretion rate of LH and its apparent irreversible metabolic clearance rate (or half-life) are essentially invariant over different stages of the menstrual cycle (Sollenberger et al., 1990). In the somatotropic axis, alterations in both the frequency and amplitude of episodic GH secretory bursts as well as substantial variations in the half-life of endogenous GH can be recognized in different states of health and disease (Mauras et al., 1987; Hartman et al., 1990; Veldhuis et al., 1990a). For example, in puberty, there is a several-fold increase in the mass of GH secreted per burst with no change in the apparent half-life of endogenous GH, whereas in healthy aging and obesity the frequency and amplitude of GH secretory bursts and the half-life of GH decrease substantially (Mauras et al., 1987; Veldhuis et al., 1990a; Iranmanesh et al., 1991). Conversely, in hyperthyroidism, the number of detectable GH secretory bursts increases (Iranmanesh et al., 1990).

A disadvantage of the waveform-specific approach to deconvolution is the need to assume a plausible waveform for the underlying secretory event. In some cases, a secretory-burst waveform cannot be surmised readily. Alternatively, a putative secretion waveform identified under physiological conditions might undergo distortion in nonphysiological states. Consequently, attention has also been directed to waveform-independent procedures to evaluate in vivo glandular secretion. Such methodologies in general require a priori knowledge of the hormone half-life. Despite this potential limitation, model-independent deconvolution has fostered important new insights into the function of endocrine glands in vivo.

Waveform-independent deconvolution has been used to evaluate the mode of release of many hormones in various pathophysiological states. For example, we have observed that aldosterone, cortisol, renin, insulin, parathyroid hormone, and all anterior pituitary hormones are released in discrete bursts often without an inter-peak 'basal' (constitutive) pattern of secretion (Veldhuis and Johnson, 1990; Veldhuis et al., 1990c). Earlier studies utilizing both in vivo and in vitro techniques have shown pulsatile patterns of insulin secretion characterized by nearly uniform periodicities (Turner et al., 1972; Polonsky et al., 1985). More recently, departures from normal

secretory patterns have been observed in a variety of clinical pathophysiological settings, including primary aldosteronism, Cushing's disease, acromegaly, diabetes mellitus, fasting, obesity, hypo- or hyperthyroidism, etc. In cases of tumoral hypersecretion, a markedly abnormal pattern of presumptively constitutive hormone secretion can occur, as shown in Fig. 4 for GH release in an acromegalic subject. The patient's GH-secreting pituitary tumor was marked by high rates of basal GH secretion with low-amplitude, high-frequency 'stuttering' bursts of GH release superimposed.

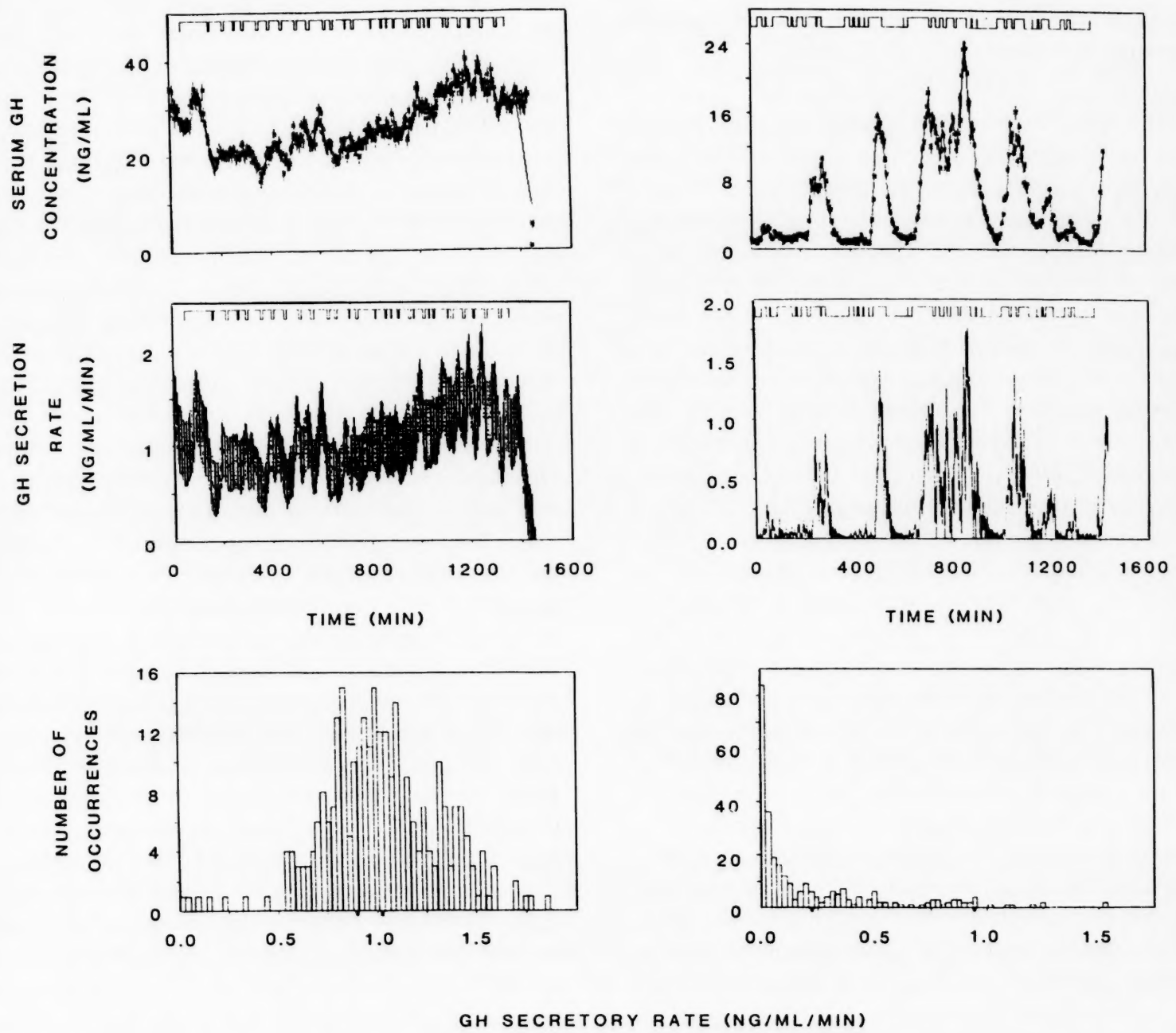


Fig. 4. Apparent increase in basal growth-hormone (GH) secretion in a patient with a GH-secreting pituitary tumor (left) compared with normal (right). The top panels give the observed plasma GH concentration data and the calculated reconvolution fit. The profiles in the middle panels are calculated sample secretory rates (and their 63% confidence limits) based on a secretion waveform-independent deconvolution algorithm. The bottom panels give the distributions (histograms) of sample GH secretory rates. Note that the tumor patient exhibited a markedly different distribution of individual sample secretory rates (middle subpanels). The patient's pathological GH profile also exhibits a high baseline or tonic secretory mode with small pulsatile episodes superimposed. For deconvolution, purposes, a biexponential kinetic function was assumed to consist of GH half-lives of 3.5 ± 1.0 min (first component) and 21 ± 1.1 min (second component) with the second component constituting 67% of the total amplitude. Data were provided by Drs. Mark Hartman and Michael Thorner, and derive from blood sampled every 5 min for 24 h for the subsequent immunoradiometric assay of serum GH concentrations in duplicate.

Such a pattern is entirely distinct from that recognized in healthy individuals under various metabolic conditions, e.g. in fasting or fed states, diurnally, during sleep, etc. Consequently, marked dysregulation of somatotrope secretory function can be inferred in this tumoral setting.

What is the functional significance or episodic hormone secretion?

The generality of an episodic (rather than a time-invariant or continuous) mode of hormone secretion suggests possible physiological importance to this temporal organization. Several significant implications of episodic hormone secretion can be considered. First, episodic endocrine glandular signaling can prevent target-cell downregulation or desensitization, which otherwise might occur if initially responsive tissues were exposed to tonic or time-invariant stimuli. Desensitization in the face of a constant stimulus is particularly noteworthy in the case of the gonadotropic axis, in which an unchanging GnRH stimulus promotes substantial downregulation of gonadotroph secretory function (Marshall and Kelch, 1986), e.g. LH beta subunit gene expression is inhibited by constant GnRH stimulation. This downregulation response has been exploited therapeutically in the development and use of GnRH agonists that can deliver an unremitting GnRH stimulus and thereby achieve a decrease in gonadotropin secretion in relevant pathophysiological settings, e.g. GnRH agonist therapy of precocious puberty, prostatic cancer, etc. (Arnaud et al., 1986). However, an unvarying GnRH stimulus does not downregulate the common alpha subunit gene, thus explaining continued alpha subunit secretion despite suppression of intact LH secretion (Shupnik, 1990). Similarly, an unvarying CRH signal to corticotropic cells in the anterior pituitary gland can cause downregulation, whereas an intermittent stimulus promotes continuing secretory activity (Vierhapper and Waldhausl, 1986). In the same manner, intermittent but not continuous ACTH injections sustain both aldosterone and cortisol secretion (Seely et al., 1989). Moreover, intermittent insulin or glucagon delivery elicits more conspicuous effects on glucose metabolism than temporally constant doses of agonist (Matthews et al., 1983; Weigle et al., 1984).

A second consideration is that an intermittent mode of hormone secretion provides for economy in endocrine glandular signaling, inasmuch as occasional bursts of hormone release in the presence of slow metabolic removal of the secreted molecules can sustain plasma hormone concentrations at a reduced secretory workload (or total time involved in secretion) (Veldhuis et al., 1987). For example, hormones with very short half-lives such as ACTH and GH require a higher amplitude and/or frequency of secretory episodes to achieve mean plasma hormone concentrations equivalent to those of hormones with a longer half-life (e.g. glycoprotein hormones, such as LH, FSH, or TSH). For target tissues that respond to mean plasma hormone concentrations or nadir values, a mechanism consisting of episodic hormone secretion combined with a relatively long half-life of hormone removal can be appropriate.

Third, a pulsatile mode of hormone release permits encoding and delivery of both frequency and amplitude-specified information to the target tissue. Such a higher-order communicating system may be important to complex physiological responses such as the development, maturation, and ovulation of ovarian follicles and the consequent formation of an effective corpus luteum. Even so, recent analyses of the human menstrual cycle indicate that a constant daily LH production rate is maintained across the follicular and luteal phases. However, this invariance of the total mass of LH secreted per day is achieved by distinct mechanisms that entail modulation of LH secretory burst amplitude, frequency, and duration (Sollenberger et al., 1990). At present, the exact cellular and biochemical consequences of regulating specific LH secretory burst properties as a function of stage of the menstrual cycle are not known. Even so, alterations in the characteristics of the gonadotropin pulse signal delivered to in vitro perifused ovarian tissue can significantly influence the responsiveness of those cells (Johanson et al., 1989; Weiss et al., 1989). The molecular mechanisms of such effects are not understood. Moreover, short-term responses of follicular and luteal tissue are sufficiently rapid that nearly instantaneous correlations exist between pulses of LH and pro-

gesterone release by the corpus luteum (Procknor et al., 1986; Veldhuis et al., 1988a). A similar close temporal coupling of release exists between ACTH and cortisol as well as between ACTH and beta endorphin in vivo (Veldhuis et al., 1990b). The exact biochemical and molecular responses initiated by the episodic (rather than continuous) presentation of cortisol and progesterone to their corresponding target cells is not known currently.

A fourth possible implication of pulsatile hormone release is the coordination of various processes within the organism with external events. For example, the onset of sleep with concomitantly reduced physical activity by the organism is associated with either augmentation or suppression of hormone release. In the case of GH, recent studies utilizing deconvolution analysis and a highly intensive sampling paradigm (30 s sampling throughout the night with simultaneous EEG monitoring) have disclosed a striking temporal association between stage III and IV sleep and in vivo GH secretion in man (Holl et al., 1990). The metabolic actions of growth hormone during sleep may be significant in maintaining gluconeogenesis, an appropriate rate of lipolysis, and the conservation of body protein. Similarly, the abrupt increase in episodic cortisol release that appears prior to awakening in the human may be relevant to preparing the individual for stress-adaptive encounters in the next several hours.

A final implication of the 'on/off switch' or pulsatile mode of hormone secretion is related to the exocytotic release process. Exocytotic release

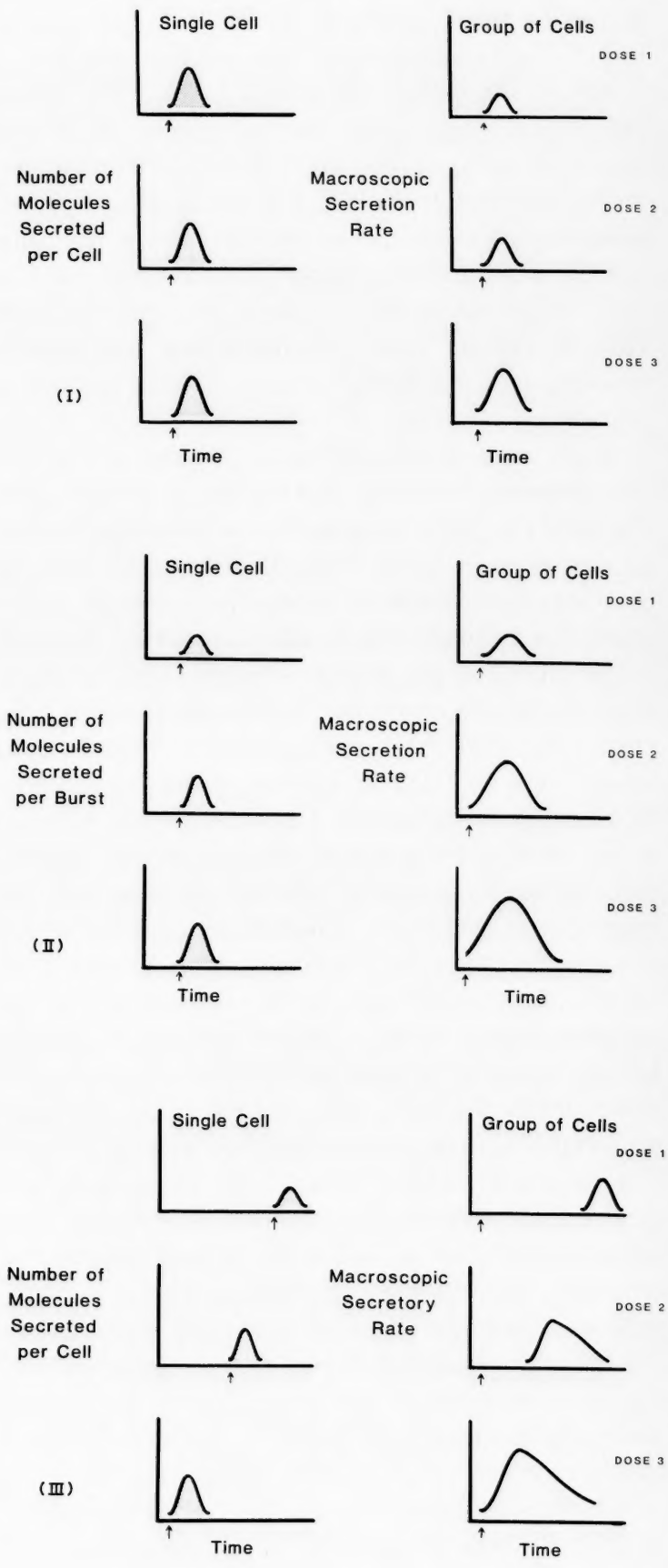


Fig. 5. Speculative models of the integration of microscopic (single-cell) and macroscopic (cell ensemble, or endocrine gland) secretion. Three putative schemata are suggested: I. Increasing agonist doses recruit a greater *number* of cells that are secreting a nearly fixed amount of hormone per cell (i.e. simple 'on-off' model). Larger macroscopic secretory output is due to the participation of more secreting cells. II. Increasing agonist doses augment the *amount* of hormone (number of molecules) secreted per cell with less or effect on the number of cells participating in secretion. Heightened macroscopic secretory output is attributable to higher secretory rates attained by every cell. III. Increasing agonist doses abbreviate the *latency* (time to onset) of secretion. In this schema, higher agonist doses shorten the time to onset, and time to maximum, of the macroscopic secretory event, and may also alter the time-shape and/or amplitude of the secretion event. In models I and II, the amplitude and duration of the macroscopic secretory event are enhanced by increasing agonist doses. In model III, the secretory waveform can be altered in shape as well as area (integrated secretion, or total mass of hormone released). Not shown are: (i) an increased frequency of agonist exposure, which can also amplify total secretion over 24 h; and (ii) various combinations of I–III and (i) above.

is clearly a feature of anterior pituitary hormone secretion, and also that of insulin, PTH, and posterior pituitary hormones. Since steroid hormones are not known to coalesce within distinct granules, but rather appear to diffuse out of the cell, any burst-like mode of steroid secretion can be attributed to the episodicity of the tropic hormone, which itself is discharged into the bloodstream by exocytotic processes (e.g. renin from the juxtaglomerular apparatus to activate aldosterone secretory bursts; ACTH from corticotropes triggering cortisol secretion; LH from gonadotropes promoting progesterone release; FSH from gonadotropes stimulating estradiol production, etc.).

How is cellular secretion integrated into macroscopic hormone release?

Each week produces more information about the subcellular control of endocrine processes from the level of gene transcription to granule exocytosis. However, further insights also are required into the coordinated integration of secretory activities. For example, we need to understand much better the coupling mechanisms that link the triggering stimulus (whether autocrine, paracrine or endocrine) and the resultant secretory impulse in single cells and cell ensembles. Novel paradigms that integrate both the independent and interacting time courses of subcellular, cellular, and in vivo glandular secretory control systems will be required to achieve a better knowledge of endocrine signaling in health and disease. In addition, observations on single-cell secretion need to be integrated into cogent explanations of macroscopic secretion by cell ensembles or intact endocrine glands. Possible speculative schemes for such integrative formulations are shown in Fig. 5. New concepts will require buttressing by informative and quantitative models of hormone secretion to provide testable hypotheses, explicit numerical representations of the constructs involved, and the generation of new ideas to complement the existing framework of experimental endocrinology.

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Review

Polyamines and mammalian hormones

Part I: Biosynthesis, interconversion and hormone effects

Giuseppe Scalabrino, Erna C. Lorenzini and Maria E. Ferioli

Institute of General Pathology and C.N.R. Center for Research in Cell Pathology, University of Milan, 20133 Milan, Italy

Key words: Polyamines; Mammalian hormones; Biosynthesis; Interconversion pathway; Biosynthetic pathway

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I cervelli atti più alla loquacità ed ostentazione, che alla specolazione ed investigazione dell'opere più segrete di natura; li quali, prima che ridursi a profferir quella savia ingenua e modesta parola '*Non lo so*', scorrono a lasciarsi uscir di bocca, ed anco della penna, qual si voglia grande esorbitanza.

G. GALILEI, *Dialogo sopra i due massimi sistemi del mondo, tolemaico e copernicano* (G. IV).

There is a mind-set more inclined to talking and boasting than to speculation and investigation of the most hidden works of Nature. Rather than giving the wise and modest response '*I do not know*', those with such a mind-set rush in and write colossal stupidities.

G. GALILEI, *Dialogue concerning the two chief world systems, Ptolemaic and Copernican* (D. IV).

Address for correspondence: G. Scalabrino, MD, Istituto di Patologia Generale, Università degli Studi di Milano, Via Mangiagalli 31, 20133 Milan, Italy.

1. Preamble

Polyamines are organic cations of low molecular weight ubiquitously present in all types of

living structure, mammalian cells, prokaryotic cells, viruses, bacteriophages, yeasts and plants (Heby, 1981; Scalabrino and Ferioli, 1981, 1982; Goyns, 1982; Hopkins and Manchester, 1982; Pegg and McCann, 1982; Allen, 1983; Tabor and Tabor, 1984, 1985; Pegg, 1986, 1988). The main natural polyamines in mammals are putrescine, spermidine and spermine (with concentrations of spermidine and spermine higher than that of putrescine). Cadaverine is only occasionally present in living mammalian cells in tiny amounts (Scalabrino and Ferioli, 1981). The structural formulae of these four polyamines are presented in Fig. 1 (panel *A*).

Historically speaking, interest in polyamines and their biosynthetic enzymes in living cells was stimulated by observations that both cell growth and cell duplication in both eukaryotes and prokaryotes require the presence and biosynthesis of polyamines (for reviews, see Scalabrino and Ferioli, 1981, 1982, 1989; Tabor and Tabor, 1984, 1985; Canellakis et al., 1985; Luk and Casero, 1987; Pegg, 1988). In addition, there is accumulation of polyamines in mammalian organs and/or tissues involved in hyperplastic or hypertrophic growth processes as well as in neoplastic transformation and/or in neoplastic growth (for reviews, see Scalabrino and Ferioli, 1981, 1982, 1989; Tabor and Tabor, 1984, 1985; Luk and Casero, 1987; Pegg, 1988). However, it is now clear that polyamines also have regulatory functions of importance in various fields of cellular biochemistry, functions apparently quite unrelated to their functions in the growth and differentiation of mam-

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Abbreviations:
ADA = adenosine deaminase (EC 3.5.4.4)
ACTH = adrenocorticotropic hormone
ADRX = adrenalectomized animals
AVP = arginine vasopressin
BHK = baby hamster kidney
CCK = cholecystokinin
CG = chorionic gonadotropin
CHO = Chinese hamster ovary
CT = calcitonin
dec-SAM = decarboxylated *S*-adenosyl-L-methionine
DHT = dihydrotestosterone
EGF = epidermal growth factor
EPI = epinephrine
FSH = follicle-stimulating hormone
GH = growth hormone
HYPOX = hypophysectomized animals
HYPOX-ADRX = hypophysectomized-adrenalectomized animals
HYPOX-PARATHYRX-THYRX = hypophysectomized-parathyroidectomized-thyroidectomized animals
LH = luteinizing hormone
LHRH = luteinizing hormone-releasing hormone
LVP = lysine vasopressin
α-MSH = melanocyte-stimulating hormone
MTA = 5′-methylthioadenosine
MTA-Pase = 5′-methylthioadenosine-phosphorylase (EC 2.4.2.28)
N-EPI = norepinephrine
ODC = ornithine decarboxylase (EC 4.1.1.17)
ORCHX = orchiectomized animals
OVX = ovariectomized animals
OVX-HYSTX = ovariectomized-hysterectomized animals
PA = polyamines
PANCRX = pancreatectomized animals
PAO = polyamine oxidase
PA-Up = polyamine uptake
PBD = polyamine biosynthetic decarboxylases
PDGF = platelet-derived growth factor
PINX = pinealectomized animals
PL = placental lactogen
PRL = prolactin
PTH = parathormone
PUT = putrescine
Ref = reference
PUT-Up = putrescine uptake
SAM = *S*-adenosyl-L-methionine
SAMDC = *S*-adenosyl-L-methionine decarboxylase (EC 4.1.1.50)
SAT = spermidine/spermine acetyltransferase
SP = spermine
Sp-B-Prot = spermine-binding protein
SPD = spermidine
SPD-SYN = spermidine synthase (EC 2.5.1.16)
SPD-Up = spermidine uptake
SP-SYN = spermine synthase (EC 2.5.1.22)
SP-Up = spermine uptake
SRIF = somatotropin release inhibiting factor
T_3 = triiodothyronine
T_4 = thyroxine
TGF-α = transforming growth factor-α
TGs = transglutaminases (*R*-glutamyl-peptide: amine γ-glutamyltransferase) (EC 2.3.2.13)
THYRX = thyroidectomized animals
TRH = thyrotropin-releasing hormone
TSH = thyroid-stimulating hormone
Vit. D_3 = 1α,25-dihydroxycholecalciferol

Symbols:
= No variation in comparison with controls
↑ Increase in comparison with controls
↓ Decrease in comparison with controls

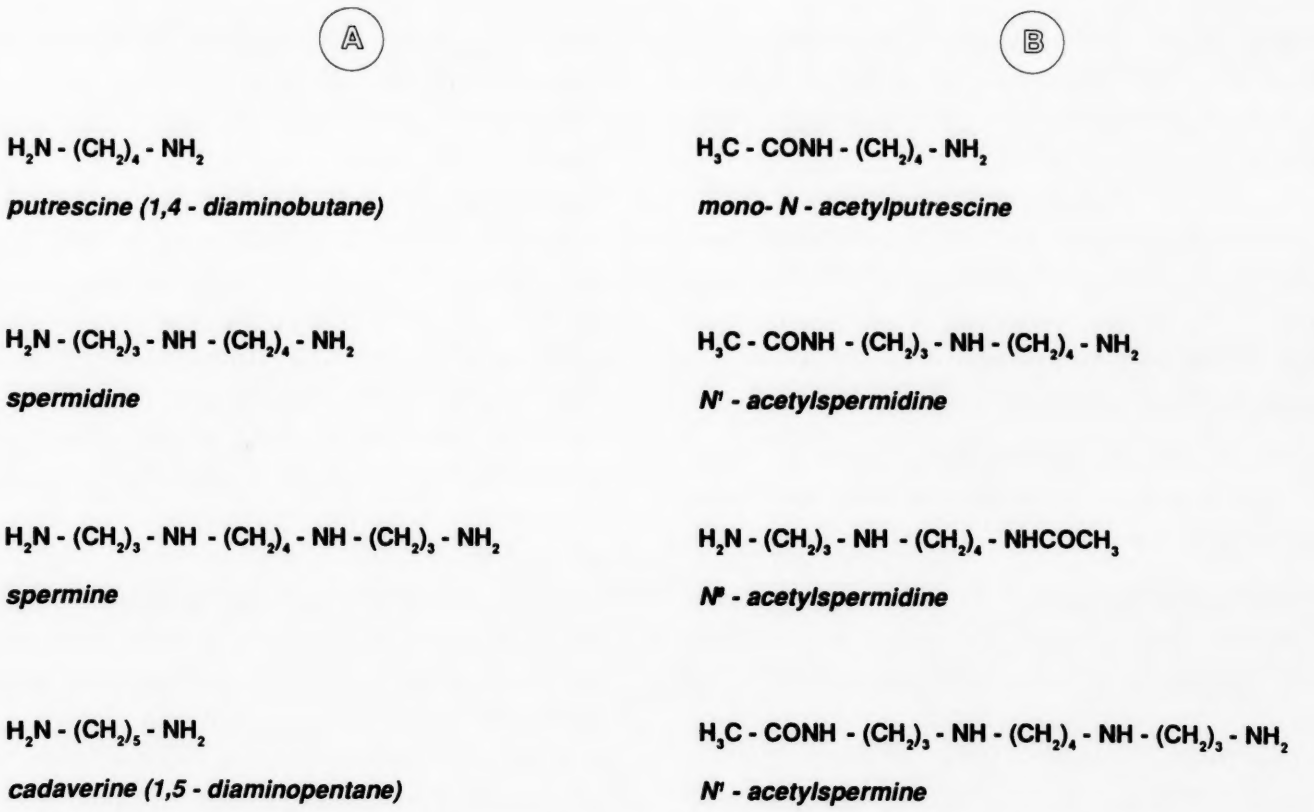
A
B
H2N - (CH2)4 - NH2
putrescine (1,4 - diaminobutane)
H3C - CONH - (CH2)4 - NH2
mono- N - acetylputrescine
H2N - (CH2)3 - NH - (CH2)4 - NH2
spermidine
H3C - CONH - (CH2)3 - NH - (CH2)4 - NH2
N1 - acetylspermidine
H2N - (CH2)3 - NH - (CH2)4 - NH - (CH2)3 - NH2
spermine
H2N - (CH2)3 - NH - (CH2)4 - NHCOCH3
N8 - acetylspermidine
H2N - (CH2)5 - NH2
cadaverine (1,5 - diaminopentane)
H3C - CONH - (CH2)3 - NH - (CH2)4 - NH - (CH2)3 - NH2
N1 - acetylspermine

Fig. 1. The structural formulae of the three main polyamines (*A*) and the structural formulae of the acetyl-conjugates of these polyamines (*B*).

malian cells (Scalabrino and Ferioli, 1981; Goyns, 1982; Tabor and Tabor, 1984) (see Section 2 of this review).

It is a firmly established concept that mammalian hormones, in both the classic and the modern sense of the word, i.e., including hormone-like molecules and cell growth factors, are among the most important regulators, positive or negative, of growth and differentiation of mammalian cells (Martin, 1985; Norman and Litwack, 1987; Cahill, 1989). Mammalian cells, in turn, have receptors for receiving hormonal messages, which are frequently concerned with, although by no means limited to, growth and differentiation (Martin, 1985; Norman and Litwack, 1987; Cahill, 1989). Thus, it is not surprising that there is much experimental evidence for various mammalian hormones (noticeably those acting on mammalian cell growth and differentiation) also regulating polyamine biosynthesis in target tissues and/or organs. The constant finding of cellular polyamine effect(s) of hormones regulating cell growth and differentiation is one piece of experimentally convincing evidence for close involvement of polyamines in growth and differentiation of mammalian cells.

The present review dealing with the connections between polyamines and hormones in mammalian cells is one tile in the broader mosaic of the connections between polyamines and growth and differentiation of mammalian cells as a whole. We have previously reviewed polyamines in mammalian tumors (Scalabrino and Ferioli, 1981, 1982, 1989), in which cell growth is generally accelerated and uncontrolled, and, in addition, polyamines in mammalian aging (Scalabrino and Ferioli, 1984), in which cell growth is controlled, but commonly slowed. In the present review we will examine the data on hormonal regulation of polyamine biosynthesis, for the possibility that polyamines also play a role in the molecular mechanism(s) of the action of mammalian hormones and cell-to-cell signals. To date, none of the major textbooks of endocrinology (Martin, 1985; Norman and Litwack, 1987; Cahill, 1989) includes even a short paragraph on this topic, nor is there currently an appropriate review. Among reviews of the physiological functions of polyamines or of particular aspects of these functions, there is only a short mention of or a brief chapter on the effects of mammalian hormones on polyamine metabolism (Williams-Ashman and Lockwood, 1970; Bach-

rach, 1973, 1980, 1984; Raina and Janne, 1975; Russell et al., 1976; Jänne et al., 1978; Rosenfeld and Barrieux, 1979; Russell and Haddox, 1979; Russell, 1980, 1981, 1983, 1985; Cochet and Chambaz, 1983; Slotkin and Bartolome, 1983; Cohen, 1985; Koenig et al., 1989b; Williams-Ashman, 1989). We therefore wish to fill this lacuna, focusing on the multifaceted problem of the connections between polyamines and hormones in mammalian cells.

2. Outline of the biochemical functions of polyamines, and details of their biosynthetic and interconversion pathways

2.1. Biochemical functions

Polyamines are present as such or as their metabolic products in mammalian cells and in mammalian physiological fluids (Heby, 1981; Scalabrino and Ferioli, 1981; Goyns, 1982; Hopkins and Manchester, 1982; Pegg and McCann, 1982; Allen, 1983; Tabor and Tabor, 1984, 1985; Pegg, 1986, 1988). Polyamines (spermidine and spermine) are usually found at millimolar concentrations in mammalian cells, whereas putrescine levels are in nanomolar concentrations. The distribution pattern varies markedly from one species to another: in a given species it differs greatly in various tissues and organs, and also varies with the growth conditions, growth rate, age, etc., of the tissue or organ (Heby, 1981; Scalabrino and Ferioli, 1981, 1984; Goyns, 1982; Hopkins and Manchester, 1982; Pegg and McCann, 1982; Allen, 1983; Tabor and Tabor, 1984, 1985; Pegg, 1986, 1988). High concentrations of polyamines are also observed in non-growing tissues, such as pancreas, prostate and lactating mammary gland that are active in protein synthesis (Oka et al., 1981; Dorn et al., 1985, 1986; Hougaard et al., 1986). Interestingly, substantial levels of polyamines have been recently demonstrated in cells producing peptide growth factors such as EGF and PDGF, or growth-stimulating peptide hormones such as GH (Hougaard et al., 1989). A substantial part of immunodetectable ODC has been demonstrated to be associated with the granules in rat heart atria, i.e., with the site of storage of atrial natriuretic peptide (Tipnis et al., 1989). The most important physiological metabolic products of polyamines in eukaryotic cells are conjugates with peptides found in some physiological fluids (Rennert et al., 1980) and the ubiquitous acetylated derivatives (for reviews, see Seiler, 1987; Seiler and Heby, 1988). The different acetylated conjugates of spermidine and spermine are the principal substrates in the pathway of polyamine interconversion (see Section 2.2 of this review). Knowledge of the polyamine interconversion is a recent, very important addition to our knowledge of the polyamine biosynthetic pathway (Seiler, 1987; Seiler and Heby, 1988). The main acetylated derivatives of the three classical polyamines (putrescine, spermidine and spermine) are shown in Fig. 1 (panel *B*).

In polyamines positive charges are distributed along a flexible chain. There is little doubt that most functions of the polyamines involve this polycationic structure, allowing electrostatic interaction with polyanions. Reflecting their structural characteristics: polyamines interact with nucleic acids, proteins and phospholipids through ionic forces and hydrogen or hydrophobic bonds (Heby, 1981; Scalabrino and Ferioli, 1981; Goyns, 1982; Hopkins and Manchester, 1982; Pegg and McCann, 1982; Allen, 1983) and thus participate in the regulation of nucleic acid and protein synthesis (Heby, 1981; Scalabrino and Ferioli, 1981; Goyns, 1982; Hopkins and Manchester, 1982; Pegg and McCann, 1982; Allen, 1983; Marton and Morris, 1987). Polyamines are known to stimulate DNA polymerases, DNA gyrase and DNA methylases. It is worthwhile to mention here that spermidine and spermine have been shown to stimulate the activity of eukaryotic type I topoisomerase (Srivenugopal and Morris, 1985) and of eukaryotic type II topoisomerase (Pommier et al., 1989). It appears now of great interest to determine the interaction of polyamines with mammalian topoisomerases, because these enzymes seem to be major components of chromosome structure in eukaryotes (Marton and Morris, 1987).

Polyamines have been shown to enhance the synthesis of RNA by DNA-dependent RNA polymerases (Heby, 1981; Scalabrino and Ferioli, 1981; Goyns, 1982; Hopkins and Manchester, 1982; Pegg and McCann, 1982; Allen, 1983). In vitro data suggest that polyamines may be general regulators of transcription by stimulating RNA polymerases I, II and III (for review, see Blair, 1985). Poly-

amines also stimulate the methylation of tRNA, increase the aminoacylation of tRNA and enhance the binding of aminoacyl tRNA to ribosomes (Raina and Jänne, 1975; Goyns, 1982; Hopkins and Manchester, 1982; Allen, 1983; Tabor and Tabor, 1984). Changes in mRNA, rRNA and tRNA induced by interaction with intracellular polyamines could thus be crucial for both regulation and the accuracy of the translation process. In this regard, the translation initiation factor eIF-4D contains the amino acid hypusine, formed by the attachment of the aminobutyl moiety of spermidine to lysine followed by hydroxylation (Park et al., 1984). In addition, almost every step in polypeptide synthesis can be stimulated in vitro by the addition of an appropriate amount of putrescine, spermidine or spermine (Raina and Jänne, 1975; Hopkins and Manchester, 1982; Tabor and Tabor, 1984). Polyamines stabilize and maintain the association of the ribosomal subunits and promote the attachment of ribosomes to endoplasmic reticulum (Raina and Jänne, 1975).

Another approach to identifying the role of the polyamines in cell growth is to isolate and to study mutants that are completely devoid of polyamines, because they lack one or more of the enzymes involved in the polyamine biosynthetic pathway. In recent years, this genetic approach has been applied to the study of polyamine functions in prokaryotes and lower eukaryotes. Such bacterial mutants have a considerably retarded rate of growth, although they can replicate indefinitely in the absence of polyamines (reviewed in Scalabrino and Ferioli, 1981; Tabor and Tabor, 1984, 1985). In contrast, yeast cell mutants that are unable to synthesize polyamines gradually stop replicating (reviewed in Tabor and Tabor, 1984, 1985). At present, relatively few mammalian cell mutants with alterations in polyamine biosynthetic enzymes are available and these have only been recently reported. A strain of Chinese hamster ovary (CHO) cells deficient in arginase activity and thus unable to synthesize ornithine has been isolated (Anehus et al., 1984). Again, other mutant CHO cells with minimal or absent ornithine decarboxylase (EC 4.1.1.17; ODC) activity have been reported (Steglich and Scheffler, 1982; Pohjanpelto et al., 1985; Steglich et al., 1985; Scheffler, 1989); ODC is the first and rate limiting enzyme in the polyamine biosynthetic pathway (Heby, 1981; Pegg and McCann, 1982; Allen, 1983;Tabor and Tabor, 1984, 1985). These alterations make such cells auxotrophic for putrescine, thus showing that mammalian cells in culture have an absolute requirement for polyamines for mitosis (Nordling and Pohjanpelto, 1990). However, some DNA synthesis can occur in these ODC-deficient CHO cells in the absence of polyamines (Nordling and Pohjanpelto, 1990). Interestingly, at the same time these cell lines also develop severe chromosomal damage, suggesting that polyamines may play a protective nuclear role (Pohjanpelto and Knuutila, 1982; Knuutila and Pohjanpelto, 1983). Nevertheless, these ODC-deficient mutants have measurable *S*-adenosyl-L-methionine decarboxylase (EC 4.1.1.50; SAMDC), the other of the two polyamine biosynthetic decarboxylases (PDB) (Heby, 1981; Scalabrino and Ferioli, 1981; Pegg and McCann, 1982; Tabor and Tabor, 1984, 1985), evidence that this part of the polyamine biosynthetic pathway is not disturbed in these cells (Pohjanpelto et al., 1985).

During the last decade, further biochemical reactions normally regulated by polyamines have been identified. There is a series of reports showing that polyamines can specifically regulate mitochondrial function and structure (Nicchitta and Williamson, 1984; Thomas et al., 1986; Gonzales-Bosch et al., 1987; Kroner, 1988; Marcote et al., 1989). The main known effects of polyamines on membranes are stabilization of membranes against lysis, promotion of membrane fusion, and inhibition of various membrane-associated enzymes and transport processes (Goyns, 1982; Hopkins and Manchester, 1982; Marton and Morris, 1987; Schuber, 1989). The mechanisms through which polyamines exert these effects are related to their polybasic nature, allowing them to adsorb to the negatively charged phospholipids of the membranes (Marton and Morris, 1987; Schuber, 1989).

In the search for other functions of polyamines in cell differentiation, much attention has been placed on some novel aspects of polyamine conjugates. It has been demonstrated that polyamines are physiological substrates for extra- and intracellular transglutaminases (*R*-glutamyl-peptide: amine γ-glutamyl transferases) (EC 2.3.2.13; TGs)

(Folk, 1980; Williams-Ashman and Canellakis, 1980; Russell and Womble, 1982; Lorand and Conrad, 1984). TGs appear to be responsible for physiologically important cross-linking of structural proteins, such as in fibrin clot polymerization, seminal plug formation (extracellular TGs) and in the stabilization of the plasma membrane during the terminal differentiation of keratinocytes (intracellular TGs) (Folk, 1980; Williams-Ashman and Canellakis, 1980; Russell and Womble, 1982; Lorand and Conrad, 1984).

A polyamine active transport system distinct from those involved in the transport of amino acids is present in all mammalian cells examined so far (Rinehart and Chen, 1984; Seiler and Dezeure, 1990). The presence of an active polyamine uptake system in bovine adrenocortical cells has been recently demonstrated (Feige and Chambaz, 1985). Mutant mammalian cell lines with decreased polyamine transport have been described (Mandel and Flintoff, 1978). The biological significance of this transport system is not fully understood, because it is intracellular synthesis that provides the cells with polyamines, and extracellular polyamine concentrations are usually small. The polyamine uptake system is not strictly specific, because a variety of polyamine analogues have been shown to be transported by this system (Rinehart and Chen, 1984; Seiler and Dezeure, 1990).

Finally, the biosynthesis of polyamines is modulated by cyclic nucleotides (see the second part of this review: Scalabrino and Lorenzini, 1991). In addition, polyamines influence the cellular content of cyclic nucleotides and may modulate certain protein phosphorylation reactions through activation of some sensitive protein kinases (Hunter, 1987) and/or modification of protein substrate conformation (Cochet and Chambaz, 1983; Cohen, 1985). Since both cyclic nucleotides and protein kinases are tightly connected to hormone effects, both topics will be discussed in depth in the second part of the current review.

For additional details on the biochemical functions of polyamines, the reader is referred to other more detailed reviews (Pegg and McCann, 1982; Scalabrino and Ferioli, 1982; Tabor and Tabor, 1984, 1985; Canellakis et al., 1985; Pegg, 1986, 1988; Luk and Casero, 1987).

2.2. Biosynthetic and interconversion pathways

2.2.1. Biosynthetic pathway

A variety of studies have clearly demonstrated that concentrations of polyamines inside both eukaryotic cells and prokaryotic cells are tightly coupled to the functional status and requirements of the cell (Heby, 1981; Goyns, 1982; Hopkins and Manchester, 1982; Pegg and McCann, 1982; Allen, 1983; Canellakis et al., 1985; Luk and Casero, 1987). The polyamine biosynthetic pathway in mammals is shown on the left side of Fig. 2. Mammalian cells produce putrescine uniquely by the irreversible decarboxylation of ornithine by ODC (Scalabrino and Ferioli, 1981; Tabor and Tabor, 1984, 1985; Canellakis et al., 1985; Pegg, 1986, 1988; Luk and Casero, 1987). The substrate ornithine may be derived from plasma or intracellular arginine by the action of arginase (EC 3.5.3.1). Arginase is most abundant in the liver of ureotelic vertebrates, although many other organs (e.g., kidney, brain, mammary gland) or cells (e.g., erythrocytes, leucocytes) contain arginase but lack the capacity to synthesize arginine. Putrescine not only serves as a substrate for spermidine synthesis, but also as the final product in the pathway of polyamine interconversion, in which spermidine and spermine are back-converted to putrescine (vide infra). In the polyamine biosynthetic pathway, putrescine is then converted into spermidine by the aminopropyl transferase spermidine synthase (EC 2.5.1.16) (Heby, 1981; Hopkins and Manchester, 1982; Pegg and McCann, 1982). A second aminopropyl transferase, termed spermine synthase (EC 2.5.1.22), adds another propylamine moiety to spermidine, forming spermine (Goyns, 1982; Hopkins and Manchester, 1982; Allen, 1983). The source of these propylamine groups is decarboxylated *S*-adenosyl-L-methionine (dec-SAM), which is produced by the irreversible action of SAMDC on *S*-adenosyl-L-methionine (SAM). SAM thus serves both as the precursor in the polyamine biosynthetic pathway and as a substrate for many transmethylation reactions, and SAMDC channels SAM into the polyamine biosynthetic pathway, withdrawing it from transmethylation reactions. Indeed, once SAM has been decarboxylated, it is committed to the polyamine biosynthetic pathway, since dec-SAM is virtually

inactive as a methyltransferase substrate (Scalabrino and Ferioli, 1981; Hopkins and Manchester, 1982; Pegg and McCann, 1982). Therefore, the amino acids that are the necessary precursors for polyamine biosynthesis are arginine and methionine. Another product of no lesser biological importance than the triamine and the tetraamine is 5′-methylthioadenosine (MTA), which is formed in both the aminopropyl transferase reactions (see again the left part of Fig. 2) stoichiometrically with spermidine and spermine. MTA, however, is very rapidly split by MTA-phosphorylase (EC 2.4.2.28) to produce 5′-methylthioribose-1-phosphate; adenine is then returned to the purine nucleoside pool and the 5′-methylthioribose-1-phosphate back converted to methionine (Heby, 1981; Scalabrino and Ferioli, 1981; Goyns, 1982; Hopkins and Manchester, 1982; Pegg and McCann, 1982; Allen, 1983; Tabor and Tabor, 1984, 1985; Pegg, 1986, 1988). Many studies (reviewed in Scalabrino and Ferioli, 1981; Williams-Ashman et al., 1982) have demonstrated that MTA strongly inhibits growth and differentiation of mammalian cells. It is therefore clear that polyamine biosynthesis simultaneously provides the cells with two types of factors that regulate growth and differentiation of mammalian cells in opposite directions, with the positive regulators being polyamines, and the negative regulator MTA.

2.2.2. Interconversion pathway

The spermidine synthase and the spermine synthase reactions are irreversible. However, only in the last decade has it been demonstrated that

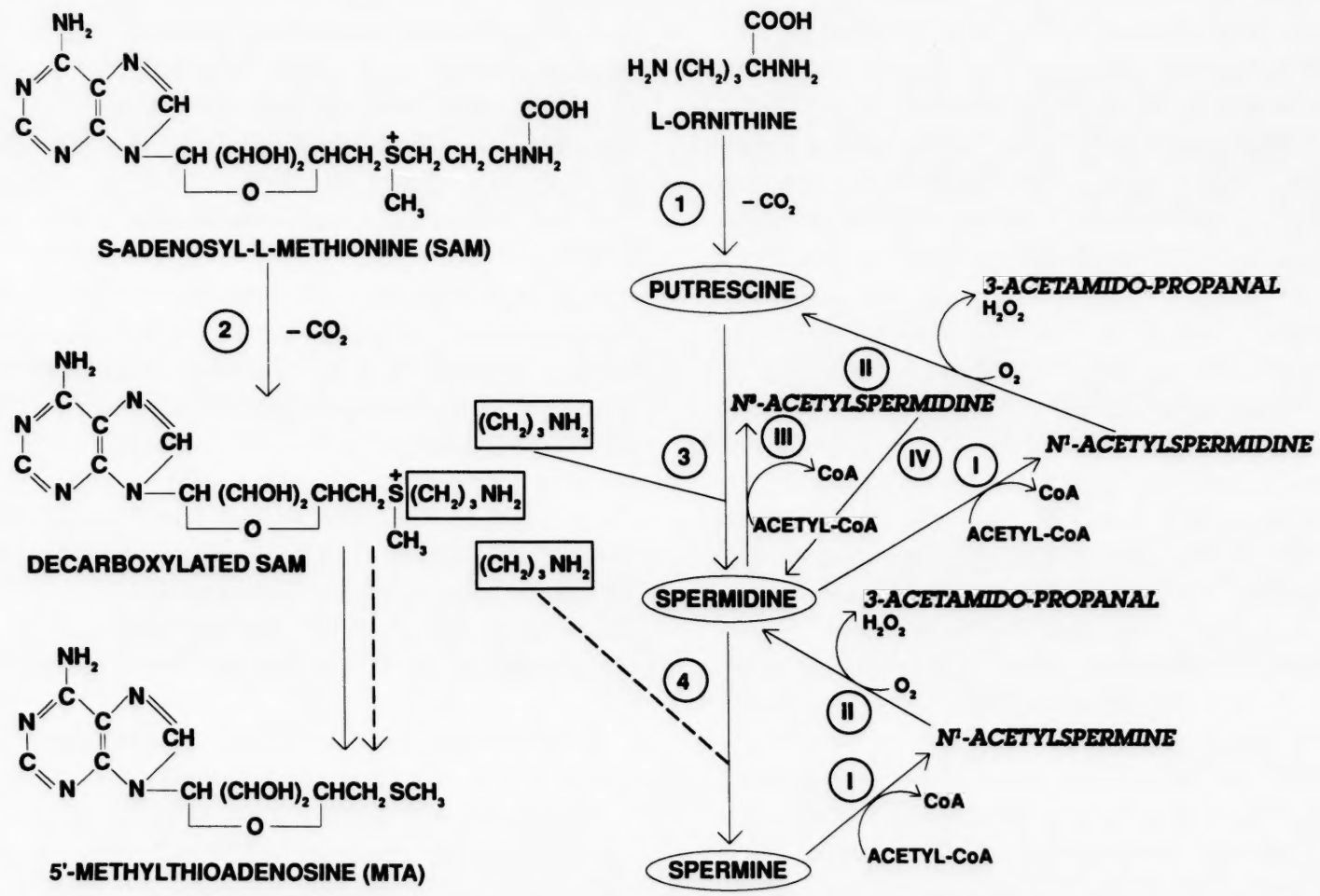

Fig. 2. The pathway of polyamine biosynthesis in mammalian cells is shown on the left and that of polyamine interconversion on the right. The enzymes involved in the biosynthetic pathway are: (1) L-ornithine carboxy-lyase (ornithine decarboxylase) (EC 4.1.1.17); (2) *S*-adenosyl-L-methionine decarboxylase (EC 4.1.1.50); (3) spermidine synthase (EC 2.5.1.16); (4) spermine synthase (EC 2.5.1.22). The enzymes involved in the interconversion pathway are: (I) acetyl-CoA : spermidine/spermine N^1-acetyltransferase (cytosolic); (II) polyamine oxidase; (III) acetyl-CoA : spermidine N^8-acetyltransferase (nuclear); (IV) N^8-acetylspermidine acetylhydrolase. The enzymes (I)–(IV) do not have EC numbers assigned to them.

spermine can be converted back to spermidine, and that spermidine can in turn be back converted to putrescine (Seiler, 1987; Seiler and Heby, 1988) by an interconversion pathway, in which one polyamine can be reutilized by being converted into another, as presented on the right side of Fig. 2. Essentially, these metabolic retro-conversions occur via oxidation of the polyamine acetylated derivatives. It is evident from both the metabolic schemes in Fig. 2 that the descending (viz. the biosynthetic) and ascending (viz. the interconversion) pathways can be viewed as a metabolic two-way route, consisting of two parts, the first going from putrescine to spermine, and the second from spermine to putrescine. The interconversion reactions involve two enzymes: a cytosolic spermidine/spermine N^1-acetyltranferase (c-SAT) and a polyamine oxidase (PAO) (Morgan, 1985, 1989). The former enzyme uses acetyl CoA to convert spermidine or spermine into N^1-acetylspermidine or N^1-acetylspermine. Thereafter, both these N^1-acetyl-derivatives of spermidine and spermine are good substrates for PAO (Beard et al., 1985; Seiler, 1987; Seiler and Heby, 1988), a flavin-dependent enzyme, which splits off 3-acetamidopropanal, yielding spermidine or putrescine, depending on the substrate. Although this oxidase also acts on non-acetylated polyamines in vitro, the acetylated polyamine derivatives are its true physiological substrates (Seiler, 1987, 1989; Seiler and Heby, 1988). Since PAO activity is much greater under normal conditions than that of the c-SAT, the content of the acetylated polyamines in eukaryotic cells is normally very low. Parenthetically, it has to be noted that spermidine synthase and spermine synthase are usually present in mammalian cells and tissues at activity levels much higher than those of PBD and c-SAT (Seiler, 1990). Furthermore, the so-called nuclear acetyltransferase (n-SAT) can acetylate all three main polyamines, but acts preferentially on spermidine, with the K_m for this substrate much lower than those for putrescine and spermidine (Seiler, 1987, 1989; Seiler and Heby, 1988). Monoacetylation by n-SAT is in the N^8-position for spermidine, but not for spermine, since the latter is a symmetrical molecule. The only known fate of this compound is deacetylation, which regenerates spermidine. The acetylase/oxidase system may act as a regulatory system to reduce the intracellular content of spermidine and spermine when they become too high, by back converting them to putrescine and spermidine. From this point of view, the interconversion pathway of polyamines resembles the interconversion pathways for purine ribonucleotides (the so-called salvage pathways for purine ribonucleotide biosynthesis from purine bases or from their ribonucleotides), which also serve to balance the intracellular levels of these purine derivatives. Like uric acid, spermidine and spermine can be toxic at high intracellular concentrations for both eukaryotic and prokaryotic cells; cytotoxic and cytostatic products, an iminoaldehyde and an iminodialdehyde, arise from oxidative catabolism of the triamine and tetraamine by amine oxidases (reviewed in Scalabrino and Ferioli, 1981). Thus, the stimulatory effects of polyamines on cell growth and differentiation may be limited by the opposite (i.e., inhibitory) effects of both MTA and these aliphatic aldehydes on cell growth and differentiation. The biological effects of this biochemical system as a whole may be depicted as a three-cornered hat, in which polyamines, MTA and aliphatic aldehydes are integrated with each other (Scalabrino and Ferioli, 1981).

The excreted forms of polyamines in mammals and polyamines in mammalian physiological fluids will be omitted from this introduction, since they are not directly concerned in the polyamine effects of mammalian hormones at cellular level; appropriate references can be found in the reviews cited (Heby, 1981; Scalabrino and Ferioli, 1981, 1982, 1989; Goyns, 1982; Hopkins and Manchester, 1982; Pegg and McCann, 1982; Allen, 1983; Tabor and Tabor, 1984, 1985; Canellakis et al., 1985; Pegg, 1986, 1988; Luk and Casero, 1987).

2.2.3. Mechanisms of control of polyamine biosynthetic and interconversion pathways

The biosynthetic and interconversion pathways are regulated by changes in the activities of three enzymes: ODC, SAMDC and c-SAT. All three enzymes have extremely short half-lives and are easily inducible by a variety of stimuli for cell growth and proliferation, such as growth factors (both normal and neoplastic), hormones, tumor promoters, chemical or viral carcinogens (Scala-

brino and Ferioli, 1981, 1982, 1989; Luk and Casero, 1987; Pegg, 1988). Both these properties enable cells to modulate levels of enzyme activity in response to positive or negative growth stimuli. Changes in the activity of PBD and c-SAT appear to reflect in part changes in enzyme protein synthesis in the cell (Heby, 1981; Scalabrino and Ferioli, 1981, 1982; Pegg and McCann, 1982). However, the rate of the enzyme degradation also contributes to the regulation of intracellular enzyme levels and consequently of intracellular levels of polyamines. Enhancement of PBD and c-SAT activity has been shown to be accompanied by lengthening of their half-lives (Heby, 1981; Scalabrino and Ferioli, 1981, 1982; Porter and Bergeron, 1988). Interestingly, mammalian cycling cells exhibit two peaks of ODC activity, which coincide with and G_1–S transition and mitosis (Sunkara and Rao, 1981). Whenever quiescent mammalian cells are induced to proliferate, a rapid induction of ODC activity has been observed during the early period of and G_0–S transition. All three key enzymes are cytoplasmatic; for ODC there is also evidence for its presence in the nucleus (Heby, 1981; Scalabrino and Ferioli, 1981; Goyns, 1982; Hopkins and Manchester, 1982; Pegg and McCann, 1982; Allen, 1983). The existence of multiple forms has been well documented only for ODC, in many mammalian organs in response to hormonal stimuli (see also Table 1) and under many other experimental conditions (for review, see Scalabrino and Ferioli, 1981).

PBD have well documented circadian rhythms in different rodent organs, including endocrine glands such as testis (de las Heras and Calandra, 1986) and adrenals (Ramirez-Gonzales et al., 1981), thus providing a model for studies of enzyme chronobiology. The circadian rhythm of renal ODC activity has been correlated with physiological circadian variation in plasma corticosterone (Nicholson et al., 1976). In addition, the pineal gland has been demonstrated to be an important part of the time-keeping machinery for circadian rhythms of PBD in the rat (Scalabrino et al., 1979b). In terms of the ontogeny of PBD, customarily very high ODC activity is seen during embryonal and fetal development, and high SAMDC activity only during adult life (reviewed in Scalabrino and Ferioli, 1984; Scalabrino et al., 1985) in many mammalian organs. Similar changes have also been demonstrated for particular endocrine glands, such as the rat adrenal (Ekker and Sourkes, 1987a) and the rat testis (Shubhada et al., 1989).

The regulation of mammalian ODC is very complex, and the physiological significance of such fine tuning remains to be explored. It is well known that polyamines, chiefly putrescine, strongly inhibit ODC activity in mammalian cells both in vivo and in vitro (the so-called amine repression of ODC) (Heby, 1981; Hopkins and Manchester, 1982; Pegg and McCann, 1982; Canellakis et al., 1985). In particular, putrescine appears to be of paramount importance in the regulation of the whole polyamine biosynthetic pathway in eukaryotic cells; this diamine both markedly inhibits ODC activity (feedback inhibition) and activates SAMDC (Pegg and McCann, 1982; Allen, 1983; Canellakis et al., 1985). SAMDC activation ensures a greater production of dec-SAM (which is thereafter used for spermidine and spermine synthesis) when putrescine production is increased by a stimulus to ODC activity; if this were not the case, the entire polyamine biosynthetic pathway would be blocked by the putrescine accumulation.

These opposite regulatory effects of putrescine on PBD are probably largely responsible for the coordinated responses of ODC and SAMDC to both positive and negative effectors. ODC inhibition by polyamines is accomplished through three fundamentally different mechanisms: (a) a direct influence on the translation of ODC mRNA (Hayashi, 1989), since a decrease in cellular polyamine content elicits an increase in ODC mRNA translation, while the opposite effect follows an increase in cellular polyamine content; (b) an effect on the turnover of ODC, increasing its degradation (Hayashi et al., 1985; Hayashi, 1989); (c) the synthesis of a protein specifically inhibiting ODC, called ODC-antizyme, predominantly elicited by putrescine both in vivo and in vitro (Canellakis et al., 1985; Canellakis and Hayashi, 1989; Hayashi and Canellakis, 1989). ODC-antizyme is normally present in non-dividing mammalian organs with no previous exposure to polyamines (Hayashi and Canellakis, 1989). A cDNA for ODC-antizyme has been recently cloned

(Matsufuji et al., 1990). Surprisingly, ODC-antizyme mRNA did not increase in rat liver upon administration of putrescine (Matsufuji et al., 1990). The picture is further complicated by the finding of a macromolecular inhibitor of ODC-antizyme in rat liver (Fujita et al., 1982).

In summary, the activity of mammalian ODC is regulated by the complex array of the following control mechanisms: (a) changes in the rate of gene transcription (Hayashi, 1989); (b) changes in the rate of mRNA translation (Heby and Persson, 1990); (c) feedback inhibition by polyamines (Hayashi, 1989); (d) the interconversion of multiple forms (Scalabrino and Ferioli, 1981); (e) changes in the rate of enzyme degradation (Hayashi, 1989); (f) the antizyme (Canellakis and Hayashi, 1989); Hayashi and Canellakis, 1989); (g) the inhibitor of antizyme (Hayashi and Canellakis, 1989). Some of these regulatory mechanisms have been demonstrated to be temporarily or permanently altered when mammalian cells are engaged in processes of growth, whether controlled or uncontrolled (reviewed in Scalabrino and Ferioli, 1989). In addition, the mechanism whereby the 5′-untranslated region (5′-UTR) of ODC mRNA negatively regulates ODC mRNA translation has recently been simultaneously established in three different laboratories (Grens and Scheffler, 1990; Ito et al., 1990; Manzella and Blackshear, 1990).

For SAMDC regulation, putrescine acts as a positive regulator of eukaryotic SAMDC activity both by activating the enzyme and by enhancing the formation of the active enzyme through the conversion of SAMDC protein from an inactive precursor to the active enzyme (Pegg et al., 1988; White and Morris, 1989). As opposed to putrescine, spermidine and spermine negatively regulate eukaryotic SAMDC activity (Pegg et al., 1988; White and Morris, 1989). Studies carried out on the regulation of translation of mRNAs for PBD have shown that spermidine and spermine strongly reduced the translation of the mRNA for SAMDC; surprisingly, the same polyamines were more effective than putrescine in inhibiting the translation of the mRNA for ODC (Kameji and Pegg, 1987; Pegg et al., 1988; White and Morris, 1989). These results raise the possibility that spermidine and spermine are actually more important physiological regulators of mammalian ODC (Hölltä and Pohjanpelto, 1986; Kameji and Pegg, 1987; Pegg et al., 1988), thus contesting the classical view which confers the key role in ODC control to putrescine. Therefore, the two true polyamines (spermidine and spermine) seem to control their own synthesis by affecting the synthesis of PBD at the beginning of the pathway itself. Spermidine synthase and spermine synthase appear to be negatively regulated by MTA as well as by dc-SAM, which is the limiting substrate. The main regulatory points of the polyamine biosynthetic pathway, including the main positive and negative effectors, are shown in Fig. 3.

Many important differences between the two polyamine acetyltransferases (i.e., c-SAT and n-SAT) have been demonstrated (Seiler, 1987; Seiler and Heby, 1988). One obvious difference lies in the different position on the polyamine molecule where acetylation takes place (Seiler, 1987; Seiler and Heby, 1988). The nuclear enzyme (N^8-SAT) is not inducible and it also has histones as substrate (Seiler, 1987; Seiler and Heby, 1988). The cytosol enzyme (N^1-SAT) is inducible and does not acetylate histones. Furthermore, unlike ODC, c-SAT is induced by administration of polyamines and/or their synthetic analogues (Erwing and Pegg, 1986; Seiler, 1987; Seiler and Heby, 1988).

Both human and rodent genomes contain a family of ODC-related genes (van Kranen et al., 1987; Cox et al., 1988). In the human genome, the ODC genes are located on the short arm of chromosome 2 and the long arm of chromosome 7 (Winqvist et al., 1986; Cox et al., 1988; Hsieh and Verma, 1990; Radford et al., 1990); the ODC gene on chromosome 2 is in close proximity to the oncogene N-myc (Tonin et al., 1989). Genomic ODC sequences have been reported for mouse (Coffino and Chen, 1988; Katz and Kahana, 1988) and rat (van Steeg et al., 1988; Wen et al., 1989), for CHO cells (Grens et al., 1989), and in the human (van Steeg et al., 1989; Moshier et al., 1990). As expected, the coding sequence is highly conserved, as is the general arrangement of introns and exons in the gene. The nucleotide sequence of a pseudogene for rat ODC has also been recently identified (Kanamoto et al., 1989). The ODC gene is expressed in all tissue types, but it shows a level of expression that varies substantially among dif-

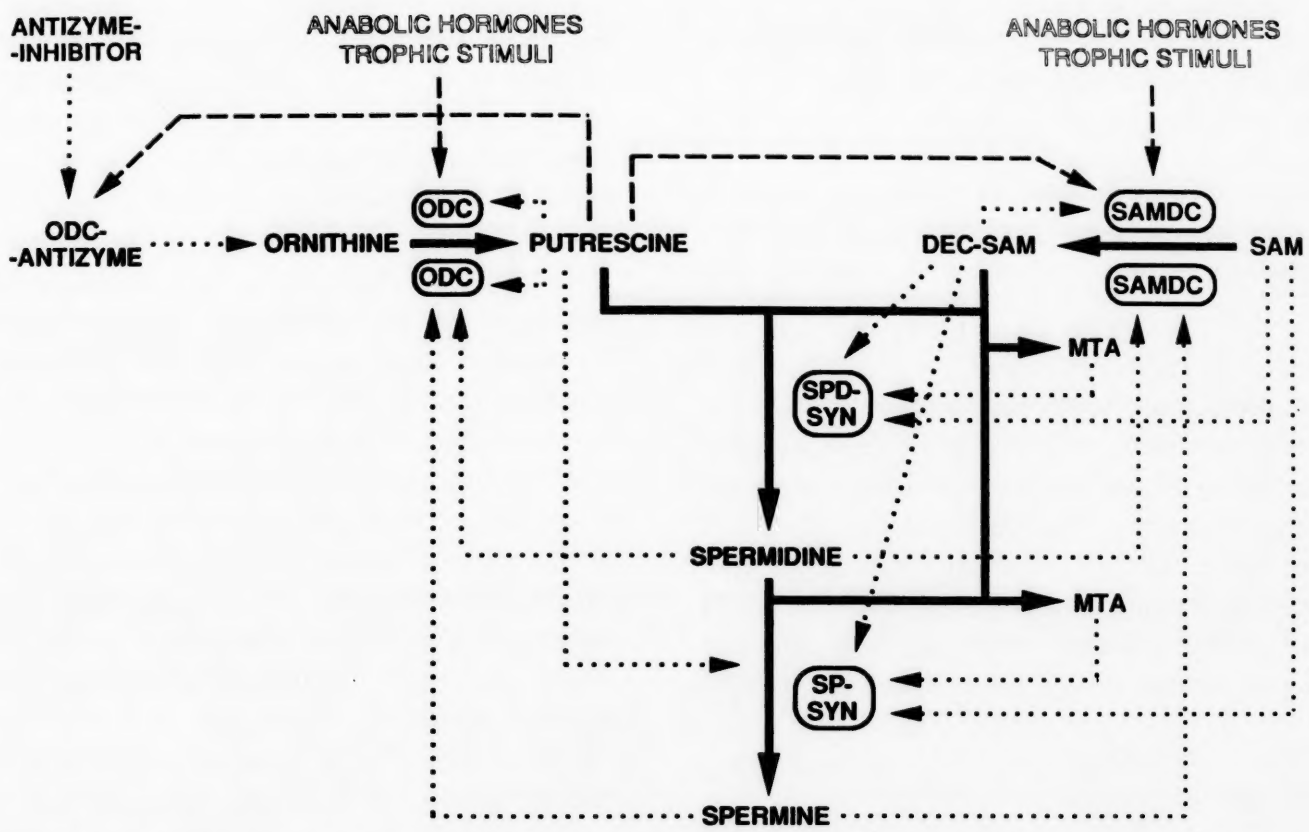

Fig. 3. Scheme showing the key points in the regulation of the polyamine biosynthetic pathway (wide arrows) in mammalian cells. In the scheme the major positively regulated nodes are indicated by dashed arrows; the major negatively regulated nodes are indicated by dotted arrows. To keep the scheme as simple as possible, only those initiating stimuli (whether hormonal or not) that enhance polyamine biosynthesis are indicated in the top part of the scheme. The reader will find hormones that inhibit polyamine biosynthesis listed in Table 1 under the appropriate organs and/or tissues and/or cultured cell lines. ODC = L-ornithine decarboxylase (EC 4.1.1.17); SAMDC = *S*-adenosyl-L-methionine decarboxylase (EC 4.1.1.50); SPD-SYN = spermidine synthase (EC 2.5.1.16); SP-SYN = spermine synthase (EC 2.5.1.22); MTA = 5′-methylthioadenosine.

ferent tissue types. The ODC gene belongs, therefore, to the so-called housekeeping gene category; as for other housekeeping genes, expression is developmentally regulated. In this regard, the expression of the ODC gene may be compared with that of the adenosine deaminase (EC 3.5.4.4; ADA) gene. ADA is present in essentially all mammalian tissues, but it shows a variable level of expression depending on the different tissues; the highest activities are found in cells of the T-lymphocyte lineage, in which ADA levels are developmentally regulated. ADA activity peaks at the cortical thymocyte stage of T-cell development and declines as T-cell maturation proceeds.

For additional details about the mechanisms and/or the molecules responsible for the regulation of the polyamine biosynthetic pathway, the reader is referred to other recent and more ample reviews (Tabor and Tabor, 1984, 1985; Canellakis et al., 1985; Hayashi et al., 1985; Pegg, 1986, 1988; Luk and Casero, 1987; Pegg et al., 1988; Porter and Bergeron, 1988; Hayashi, 1989; Kaczmarek and Kaminska, 1989; Scalabrino and Ferioli, 1989; Heby and Persson, 1990; Seiler, 1990).

Some basic concepts about the polyamine catabolic pathway and its regulation have been deliberately omitted in the present review, since there are very few studies on the hormonal regulation of the enzymes involved in the polyamine catabolism. Putrescine is metabolized to γ-aminobutyric acid in mammalian organs via diamine oxidase (Seiler, 1980); it has been recently demonstrated that γ-aminobutyric acid in the rat adrenal gland is derived mainly from putrescine and not from glutamic acid (Caron et al., 1988). This means that putrescine in the adrenal gland is more readily converted into γ-aminobutyric acid (mainly

present in the adrenal chromaffin cells) rather than into spermidine and spermine.

3. Hormones and prohormones regulating content, biosynthesis and/or interconversion of polyamines in mammalian organs, tissues and cultured cell lines

The mammalian hormones and prohormones with effects reported on the biosynthesis and/or interconversion of polyamines in different mammalian organs, tissues and cultured cell lines are listed in Table 1. For each hormone or prohormone, the organ(s), tissue(s) or cell line(s) in which polyamine effect(s) were studied, and the different polyamine effect(s) reported, are listed.

3.1. Discussion of Table 1

Historically speaking, research on the hormonal regulation of polyamine biosynthesis began with investigations of the effects of hormones on their classical target organs (Williams-Ashman and Lockwood, 1970; Bachrach, 1973, 1980, 1984; Raina and Jänne, 1975; Russell et al., 1976; Jänne et al., 1978; Rosenfeld and Barrieux, 1979; Russell and Haddox, 1979; Russell, 1980, 1981, 1983, 1985; Cochet and Chambaz, 1983; Slotkin and Bartolome, 1983; Cohen, 1985). In contrast, the biological and biochemical parameters used to date to define a target for a given hormone never have included polyamine effects. Table 1 shows that in target organs and/or tissues, polyamine biosynthesis and/or content are regulated by hormones or prohormones, and that hormones have no polyamine effects in many organs and/or tissues classically not considered targets for these hormones. In other cases, however, polyamine biosynthesis and/or content in organs not currently considered classical target organs do respond to certain hormones, suggesting that the definition of a target for a given hormone should be widened.

It is also clear from Table 1 that the regulation of polyamine biosynthesis (especially of PBD) and/or content in a given tissue is multihormonal and consequently integrated. Finally, the presence of polyamine effect(s) does not depend on the chemical nature of the hormone nor on the mechanisms of action. Both the major membrane receptor-mediated signals (i.e., polypeptide hormones, neurotransmitters) and the major intracellular receptor-mediated hormones (i.e., steroid and thyroid hormones) are able to modulate polyamine levels in various mammalian tissues.

There are a number of particular points to be made about regulation of eukaryotic PDB by mammalian hormones. First, hormonal induction of PBD observed in vivo has also been observed in isolated organs and in cultured cells. Second, in most of the experimental models used so far, the induction of these enzyme activities by hormones appears to be the result of the synthesis of new enzyme proteins and not of activation of preexisting enzyme molecules, since it is greatly reduced by concomitant addition of inhibitors of protein or RNA synthesis (for review, see Scalabrino and Ferioli, 1981). Third, the mechanism(s) underlying the induction of PBD by hormones is still not completely clarified. cAMP has often been claimed to be mandatorily involved in induction of these enzymes, especially that of ODC. This topic will be discussed in depth in the second part of the current review (Scalabrino and Lorenzini, 1991).

To the best of our knowledge, there are two reports showing a possible hormonal regulation of ODC-antizyme synthesis. It has been demonstrated that prolactin stimulated ODC activity in lymphoma cells and concomitantly induced a decrease in the ODC-antizyme presence (Richards et al., 1986). Conversely, it has been demonstrated that the amount of ODC-antizyme was increased in rat thymus after treatment with dexamethasone, i.e., when ODC activity decreased (Peng et al., 1989).

In summary, the crucial points to emerge from Table 1 are as follows:

(a) the ability to affect polyamine biosynthesis in mammalian organs and/or tissues is common to all mammalian hormones, but each hormone is active in some tissues only;

(b) the ability of some organs to respond to hormones in terms of modulation of polyamine biosynthesis is sometimes much broader than, and therefore does not always correspond with, the ability to mount a classical target tissue response;

(c) the connection between presumptive major (i.e., classical) effects and minor (i.e., the poly-

TABLE 1

EFFECTS OF MAMMALIAN HORMONES AND/OR PROHORMONES ON CONTENT AND/OR BIOSYNTHESIS AND/OR INTERCONVERSION OF POLYAMINES OF MAMMALIAN ORGANS, TISSUES AND CULTURED CELL LINES

Hormone or prohormone	Organ(s) and/or tissue(s) and/or cultured cells	Polyamine effect(s)	Remarks	Ref. No.
LHRH	Adenohypophysis	ODC =	OVX rat	[316]
	BHK cells	ODC =		[206]
	Testis (T); Leydig (L) cells; seminiferous (S) tubules	ODC-T↑; ODC-L↑; ODC-S =	Immature rat	[220,221,224]
	Heart	ODC↑	Neonatal mouse	[179]
	Ovary	ODC↑	Immature rat	[153]
	Uterus	ODC =	OVX rat	[330]
TRH	Adenohypophysis	ODC =	OVX rat	[316]
		ODC-R = ; ODC-C↑	Rat (R); chick (C)	[56]
	Prostate	ODC =	Rat	[264]
	Thyroid	ODC↑; SAMDC↑	Rat	[236]
SRIF	Liver	ODC =	Rat	[57]
α-MSH	Lung	ODC↑	Rat	[176]
ACTH	Brain; liver	PUT↑; SPD↑	Mouse	[450]
	Brain	ODC =	Rat	[357]
	Thyroid	ODC =	Rat	[343,485]
	Adrenals	ODC↑; SAMDC↑; cSAT↑	HYPOX rat	[75,77,197, 198,342]
	Adrenal medulla (M); adrenal cortex (C)	ODC-M = ; ODC-C↑	HYPOX rat	[3]
	Ovary	ODC =	Immature rat	[273]
	Thymus	ODC = ; SAMDC =	HYPOX-ADRX rat	[383]
	Kidney	ODC↑	HYPOX or HYPOX-ADRX rat	[384]
	Kidney (cortex; medulla)	ODC↑	Rat	[200]
	Lung	ODC↑	Rat	[176]
	Adrenocortical cells	ODC↑; PA↑; PUT-Up↑; SP-Up↑	Bovine	[86]
	Sertoli cells	ODC =	Bovine	[91]
TSH	Thyroid	ODC↑; SAMDC↑	Intact or HYPOX rat	[58,92,95, 236,343, 483,485]
		ODC↑	Mouse	[92,483]
	Incubated thyroid	ODC↑; PUT↑; SPD = ; SP =	Intact or HYPOX rat	[94,95,390, 391]
		ODC↑; PUT↑	Mouse	[93,96]
	Thyroid slices	ODC↑	Dog	[249]
		ODC↑	Bovine	[424]
	Thymus; spleen	ODC =	HYPOX rat	[378]
	Kidney	ODC =	HYPOX rat	[384]
	Ovary	ODC↑	Immature rat	[273]
	Liver; kidney; adrenals	ODC =	Rat	[343]
GH	Liver	ODC↑; PUT↑; SPD = ; SP =	Rat	[129,148, 283,341]
		PUT = ; SPD↑; SP =	Rat	[147]

TABLE 1 (continued)

Hormone or prohormone	Organ(s) and/or tissue(s) and/or cultured cells	Polyamine effect(s)	Remarks	Ref. No.
GH	Liver	ODC↑	Rat	[84,127,130, 142,257,325, 355,365,366, 376,449]
		SPD-SYN↑; SP-SYN↑	Rat	[148]
		PUT↑; c-SAT↑; SPD=; SP=	Rat	[233]
		ODC↑	ADRX cat	[149,295,341]
		ODC↑	Neonatal rat	[33,107,136]
		ODC=	Fetal rat	[136]
		ODC↑	Rat pup	[82,184]
		ODC↑	Weanling rat	[34,366]
		ODC↑; SPD↑; SP=	HYPOX rat	[127,182,269, 283,341,421]
		ODC↑; SAMDC↑; $t_{1/2}$ ODC↑; PUT↑	Young rat	[107,329, 366,367]
	Regenerating liver	ODC↑	Intact or HYPOX rat	[84,420]
	Liver	ODC↑	Young or aged mouse	[146]
		ODC↑	Immature mouse	[262]
	Perfused liver	ODC↑; SAMDC=	Rat	[151]
	Hepatocytes	ODC=	Fetal rat	[364]
		ODC=	Rat	[451]
		ODC↓	Rat	[394]
		ODC-I↑; ODC-H↑; PUT-I↑	Intact (I) or HYPOX (H) rat	[172]
	Kidney	ODC↑	Rat	[27,127, 376,382]
	Kidney (cortex (C); medulla (M))	ODC-C↑; ODC-M↑	Rat	[200]
	Kidney	ODC-N=; ODC-Y↑	Neonatal (N) or young (Y) rat	[107]
		ODC↑	HYPOX rat	[127,269,382, 384,421]
		ODC↑	HYPOX or ADRX rat	[384]
		ODC↑	Immature mouse	[262]
	BHK cells	ODC=		[242]
	Brain	ODC↑	Rat	[142,203,356]
		ODC↑	Neonatal rat	[33,184,355]
		ODC=	THYRX rat	[356]
		ODC=	Neonatal or fetal rat	[136]
		ODC↑	Immature mouse	[262]
	Brain cells	ODC↑	Fetal rat	[481]
	Embryonic brain	ODC=	Mouse	[20]
	Superior cervical ganglia	ODC=	Young rat	[214]
	Mammary gland explants	ODC↑	Pregnant mouse	[346]
	Thyroid	ODC↑	Rat	[485]
		ODC=	Rat	[343]
	Adrenals	ODC↑	HYPOX rat	[197,198,269]
	Adrenal cortex	ODC↑	Weanling rat	[34]

TABLE 1 (continued)

Hormone or prohormone	Organ(s) and/or tissue(s) and/or cultured cells	Polyamine effect(s)	Remarks	Ref. No.
GH	Ovary	ODC =	Immature rat	[273]
	Heart	ODC =	Fetal or neonatal rat	[136]
		ODC↑	Neonatal mouse	[262]
		ODC↑	HYPOX rat	[421]
	Perfused heart	ODC↑	Rat	[9]
	Thymus	ODC =	Intact or HYPOX rat	[378]
		ODC↑	HYPOX rat	[421]
	Spleen	ODC↑	Intact or HYPOX rat	[378,421]
	Lung	ODC↑	HYPOX rat	[421]
	Testis	ODC↑	Neonatal mouse	[262,427]
		ODC =; SAMDC =; SPD =	HYPOX rat	[216]
	Intestine	ODC↑	Neonatal mouse	[262]
	Pelvic cartilage	ODC =	Chick embryo; in vitro	[32]
	Epidermal cells	ODC =	Chick embryo	[426]
	Ob cells	PUT =; SPD↑; SP =		[4]
	Cultured pancreatic islets	PUT↑; SPD↑; SP↑	Fetal rat	[416b]
FSH	Testis	ODC↑	Neonatal mouse	[427]
	Testis (T); Leydig (L) cells; seminiferous (S) tubules	ODC-T↑; ODC-L =; ODC-S↑	Immature rat; in vivo; in vitro	[217,218,221–224,290,334]
	Testis; isolated interstitial cells	ODC =	Rat; in vitro	[290]
	Testis	PUT↑; SPD =; SP =	Newt	[237]
	Sertoli cells	ODC↑; SPD↑; SP↑	Immature rat	[433,434]
		ODC↓	Rat	[225]
		ODC↑; PUT↑; SPD =	Bovine	[91]
	Kidney	ODC =	HYPOX rat	[384]
	Ovary	ODC↑	Immature rat	[273,411]
		ODC =	Prepubertal or adult rat	[175,419]
	Granulosa cells	ODC↑	Immature pig	[287,289,456–458,460]
	Immature (I) or mature (M) granulosa cells	ODC-I↑; ODC-M =	Pig	[285,459,461]
	CHO cells	ODC↑		[61]
LH	Ovary	ODC↑; SAMDC =	Immature or pubertal rat	[138,169,175,188,238,273,274,411,419]
		ODC↑	Pregnant rat	[138]
		ODC =	Immature rat	[196]
	CHO cells	ODC↑		[61]
	Cultured granulosa cells	ODC↑	Mature or immature pig	[285–289,456–463]
	Embryonic ovary	ODC↑	Chick; in vitro	[444]
	Thyroid	ODC =	Rat	[485]
	Kidney	ODC =	HYPOX rat	[384]
	BHK cells	ODC =		[206]
	Testis	ODC↑	Neonatal mouse	[427]
		ODC↓	Immature rat	[336]

TABLE 1 (continued)

Hormone or prohormone	Organ(s) and/or tissue(s) and/or cultured cells	Polyamine effect(s)	Remarks	Ref. No.
LH	Testis (T); Leydig (L) cells; seminiferous (S) tubules	ODC-T↑; ODC-L↑; ODC-S =	Immature rat	[217,218,221–224,334]
	Testis (T); Leydig (L) cells; interstitial (I) cells	ODC-T =; ODC-L↑; ODC-I↑	Rat; in vitro	[290–292]
	Sertoli cells	ODC↑	Bovine	[91]
	3T3 fibroblasts	ODC↑		[50]
PRL	Mammary gland explants	ODC↑; SPD↑; SP =; PA-Up =	Pregnant mouse	[163,277,344–351,477,478]
	Liver	ODC↑	Intact or HYPOX rat	[339,340c,355,376,378,379,448]
		ODC =	Fetal or neonatal rat	[33,136]
		ODC↑	Neonatal rat	[136]
	Liver slices	ODC↑	Larval tiger salamander	[137]
	Kidney	ODC↑; PUT↑; SPD =; SP =	Intact rat	[339,340c,376,448]
		ODC↑; PUT↑; SPD↓; SP =	HYPOX rat	[376,448]
		ODC =	HYPOX rat	[384]
	Brain	ODC↑	Intact or HYPOX rat	[355]
		ODC↑	Neonatal rat	[33]
		ODC =	Fetal or neonatal rat	[136]
	Adrenals	ODC↑	Intact or HYPOX rat	[339,376,448]
	Spleen	ODC↑	Intact or HYPOX rat	[339,376,378,379,448]
	Thymus	ODC↑	Intact or HYPOX rat	[339,376,378,379,448]
	Heart	ODC↑	Intact or HYPOX rat	[339,376]
		ODC =	Neonatal rat	[136]
	Lung, adipose tissue	ODC =	Rat	[339]
	Granulosa cells	ODC =	Immature pig	[457]
	Sertoli cells	ODC =	Bovine	[91]
	Testis; interstitial cells	ODC =	Rat; in vitro	[290]
AVP	Kidney	ODC↑	Neonatal and young rat	[107]
		ODC↑	Hydrated or dehydrated mouse	[298,299]
		ODC↑	HYPOX rat	[382]
		ODC↑	ORCHX mouse	[299,301]
		ODC↑	ADRX mouse	[299]
	Liver	ODC↑	Intact or ADRX rat	[78,185]
	Aorta	ODC↑	Chick	[226]
	Lung	ODC↑	Rat	[176]
	Testis; seminiferous tubules; Leydig cells	ODC↓	Immature rat	[336]
		ODC↑	Immature rat	[335]
	Swiss 3T3 cells	ODC =		[70]
	Henle's loop	ODC↑	Rat	[42]
LVP	Kidney	ODC↑	Rat	[299]
	Liver	ODC↑	Intact or ADRX rat	[78,142]

TABLE 1 (continued)

Hormone or prohormone	Organ(s) and/or tissue(s) and/or cultured cells	Polyamine effect(s)	Remarks	Ref. No.
LVP	Brain	ODC-I↑; ODC-H =	Intact (I) or HYPOX (H) rat	[142]
	3T3 cells	ODC↑		[425]
Oxytocin	Kidney	ODC =	Hydrated or dehydrated mouse	[298]
	Liver; brain	ODC =	Rat	[142]
Melatonin	Kidney	ODC =	HYPOX rat	[382]
	Thymus	ODC =; SAMDC =	PINX rat	[383]
Angiotensin II	Adrenals	ODC↓	HYPOX rat	[342]
	Brain cells	ODC =	Fetal rat	[481]
	Liver (L); brain (B); superior cervical ganglia	ODC-L↑; ODC-B↑; ODC =	Neonatal, adult rat	[113,142]
	Aorta	ODC↑	Chick	[226]
	Adrenocortical cells	PA-Up↑	Bovine	[86]
	Lung	ODC↑	Rat	[176]
	Kidney	ODC-N =; ODC-Y↑	Neonatal (N), young (Y) rat	[107]
Renin	Liver; brain	ODC↑	Rat	[10]
PL	Liver	ODC =	Neonatal rat	[33,355]
		ODC↑	Fetal or neonatal rat	[33,136,184]
	Brain	ODC =	Fetal or neonatal rat	[33,136]
		ODC↑	Neonatal rat	[33,355]
	Heart	ODC =	Fetal or neonatal rat	[136]
	Pelvic cartilage	ODC =	Chick embryo; in vitro	[32]
CG	Ovary	ODC↑; SAMDC =; PUT↑; SPD-SYN =; SP-SYN =	Rat	[165,175, 238,323]
	Testis	ODC↑	Rabbit	[482]
	Thyroid	ODC =	Rat	[343]
	Ovary	ODC↑; SAMDC↑; PUT↑; SPD =; SP↓; ODC-mRNA↑	Immature rat	[114,273, 406,472]
	Ovarian cells	ODC↑	Rat; in vitro	[319,323]
	Ovary	ODC↑	Pregnant rat	[122]
		PUT↑; SPD↑; SP↓; ODC-immunore-active cells↑	Prepubertal rat	[5,313]
		ODC↑	Rabbit	[21]
	Ovarian cells	ODC↑	Prepubertal rat	[164]
	Sertoli cells	ODC↑	Bovine	[91]
	Corpora lutea	ODC =	Pregnant ox	[205]
Insulin	Liver	ODC↑	Neonatal or adult rat	[49,82]
		ODC↑	ADRX or PANCRX rat	[170,256,295]
		ODC =	HYPOX rat	[341]
		ODC↑; SAMDC↑; PUT↑; SPD↓; SP =; c-SAT =	Chick	[108–111]
	Perfused liver	ODC↑	Intact or ADRX rat ADRX rat	[199,227]
	Regenerating liver	ODC↑; PUT↑; c-SAT =	Rat	[381b]
	Hepatocytes	ODC↑	Rat	[212,261, 394,451]

TABLE 1 (continued)

Hormone or prohormone	Organ(s) and/or tissue(s) and/or cultured cells	Polyamine effect(s)	Remarks	Ref. No.
Insulin	Hepatocytes	ODC =	Fetal rat	[364]
	Liver; brain; kidney; adrenals (A)	ODC = ; ODC-A ↓	Rat	[203,260]
	Brain	ODC ↑	Neonatal or adult rat	[357,446]
	Brain cells	ODC ↑	Fetal rat	[481]
	Cultured superior cervical ganglia	ODC ↑	Young rat	[214]
	Brain cells	ODC ↑	Chick embryo	[297]
	Brain	ODC ↑	Mouse embryo; in vitro	[20]
	Heart	ODC ↑	Neonatal rat	[14,189,445, 446,465]
	Perfused heart	ODC ↑	Rat	[9]
	Heart cells	ODC ↑	Neonatal rat	[451b]
	Small intestine	ODC ↑	Rat	[239]
	Kidney; spleen; thymus	ODC ↑	Rat	[376,378]
	Mammary gland explants	ODC ↑ ; SAMDC ↑ ; SPD-SYN ↑ ; PA ↑ ; PA-Up ↑	Pregnant or virgin mouse	[1,143,163, 275–279, 380]
	Adrenals (medulla)	SAMDC ↓	Rat	[77]
	Heart; brain (B); pancreas (P); kidney; intestinal mucosa	ODC ↑ ; ODC-B ↓ ; SAMDC ↑ ; SAMDC-B ↓ ; c-SAT-P ↑ ; PUT-P ↑ ; SPD-P ↓ ; SP-P =	Chick	[110,111]
	Testis; interstitial cells	ODC =	Rat; in vitro	[290]
	3T3 fibroblasts	ODC = ; ODC ↑ (+ asparagine or amino-isobutyric acid)		[22,135,195, 353,354]
	Fibroblasts	ODC ↑	Chick embryo	[116,418]
	L cells	ODC ↑	Mouse	[480]
	Fibroblasts	ODC ↑ ; PUT-Up ↑	Human; mouse	[71,152]
	Fibroblasts	ODC-mRNA =	Human	[157]
	Epidermal cells	ODC =	Chick embryo	[426]
	CHO cells	ODC ↑		[61]
	BHK cells	ODC ↑		[126]
	Swiss 3T3 cells	ODC ↑	Mouse	[70,271,425]
	Incubated pelvic cartilage	ODC ↑ ; $t_{1/2}$ ODC ↓	Chick embryo	[32]
Glucagon	Liver	ODC ↑	Rat	[128,256,449]
		ODC ↑	Neonatal rat	[82,83,185]
		ODC ↑	HYPOX or ADRX rat	[170,295,341]
		ODC ↑	PANCRX rat	[256]
	Regenerating liver	ODC = ; PUT ↑ ; c-SAT ↑	Rat	[381b]
	Perfused liver	ODC ↑ (+ amino acids)	Intact or ADRX rat	[227]
	Hepatocytes	ODC ↑ ; ODC ↑ (+ asparagine); ODC-mRNA ↑ ; $t_{1/2}$ ODC ↑	Rat	[160,161, 172,212]
		ODC =	Fetal rat	[364]
		ODC ↓	Rat	[394,451]

TABLE 1 (continued)

Hormone or prohormone	Organ(s) and/or tissue(s) and/or cultured cells	Polyamine effect(s)	Remarks	Ref. No.
Glucagon	Brain cells	ODC =	Fetal rat	[481]
	Thymus; spleen; brain; liver; kidney; adrenals	ODC =	Rat	[260,378]
	Kidney	ODC↑	Rat	[376]
	Stomach (S); duodenum (D); ileum (I); colon (C)	ODC-D↑; ODC-I↑; ODC-S =; ODC-C =	Rat	[400]
	Pancreas	c-SAT↑	Chick	[111]
	Testis	ODC =	Neonatal mouse	[427]
	Enterocytes	ODC =; SAMDC =	Rat	[88]
	Epidermal cells	ODC =	Chick embryo	[426]
	Heart cells	ODC↑; PA↑	Chick embryo	[51]
	Cultured superior cervical ganglia	ODC =	Young rat	[214]
CCK	Pancreas	PA↑; ODC↑	Rat	[90b,115–115d, 208b]
		ODC-mRNA↑	Rat	[338]
	Intestine	ODC =	Rat	[97]
Secretin	Pancreas	ODC↑; PUT↑; *N'*-acetylspermidine↑; c-SAT↑; SPD =; SP =	Rat	[66,115b,115c]
Caerulein	Pancreas	PA↑; ODC↑	Rat	[253,254,392]
		ODC =; PA =; c-SAT =	Rat	[66]
Bombesin	Lung	ODC↑	Rat	[176]
	Pancreas	PA↑	Rat	[392]
Pentagastrin	Stomach; duodenum; ileum; colon; oxyntic gland	ODC =; SAMDC =; PUT↓; SPD↑; SP↑; c-SAT↑; acetyl-SPD↑	Rat	[154,399–401]
	Kidney	ODC↑	HYPOX rat	[382]
	Enterocytes (crypt cells)	ODC-immunoreactive cells↑	Rat; in vivo	[155]
	Enterocytes	ODC =; SAMDC =	Rat	[88]
	Colonic mucosal explants	ODC-mRNA↑; ODC↑	Rat	[9b,226b]
	Small intestine	ODC =	Rat	[97,239]
		ODC↑	Rat	[465]
Serotonin	Kidney	ODC-H↑; ODC-HA↑	HYPOX (H) or HYPOX-ADRX (HA) rat	[382]
	Endothelial cells	ODC↑	Neonatal calf	[64]
	Lung	ODC↑	Rat	[176]
T_3	Liver	ODC↑	Intact or HYPOX rat	[47,57]
		ODC↑	THYRX rat	[173b,294]
	Regenerating liver	ODC↑	HYPOX rat	[420]
	Thyroid	ODC =	Mouse; rat	[95,343]
	Myocardium	ODC↑; $t_{1/2}$ ODC↓; ODCs; SPD↑; SP↑	Neonatal or adult rat	[15,47,48,173, 189–191,333]
	Perfused heart	ODC↑	Rat	[9]
	Brain	ODC↑	Hypothyroid rat	[362]
		ODC =	Rat	[47]

TABLE 1 (continued)

Hormone or prohormone	Organ(s) and/or tissue(s) and/or cultured cells	Polyamine effect(s)	Remarks	Ref. No.
T_3	Embryonic brain	ODC =	Mouse; in vitro	[20]
	Kidney	ODC↑	Intact or HYPOX rat	[376,382]
	Kidney cortex	ODC↑; PA↑	Mouse; in vitro	[102]
	Spleen	ODC =	Rat	[47,378]
	Thymus	ODC↓	Rat	[378]
	Testis	ODC =; SAMDC =; SPD =	Intact or HYPOX rat	[47,216]
	Soleus; gastrocnemius	ODC↓	Intact or ADRX rat	[46]
	Hepatocytes	ODC↓	Rat	[451]
		ODC =	Rat	[394]
	Pelvic cartilage	ODC↑	Chick embryo	[32]
T_4	Liver	ODC↑	HYPOX or ADRX rat	[295,341]
		ODC =	Rat	[260]
	Hepatocytes	ODC =	Fetal rat	[364]
	Ovary	ODC↓	Rat	[175]
	Cultured superior cervical ganglia	ODC =	Young rat	[214]
	Thyroid	ODC↓	Rat	[235]
		ODC =	Rat; mouse	[95]
	Myocardium	ODC↑; SAMDC↑; SPD-SYN↑; SP-SYN =; PA↑; dec-SAM↑	Rat	[303,304]
	Brain	ODC↑; SAMDC↑; PUT =; SPD↑; SP↑	Neonatal or adult rat	[7,139,362]
		ODC =	Intact or THYRX rat	[260,356]
	Kidney	ODC =	Rat; mouse; Tfm/Y mouse	[247,260]
	Adrenals	ODC↓	Rat	[260]
	Adenohypophysis	ODC↑	Chick	[56]
	Epidermal cells	ODC↑	Chick embryo	[426]
	Retina	ODC↑; SAMDC↑; PA↑	Neonatal rat	[215]
CT	Chondrocytes	ODC =	Rabbit ribs	[440]
	Osteoblasts	ODC =	Chick embryo calvaria	[209]
	Osteoclast-like cells	ODC =	Mouse calvaria	[25]
	Bone cells	ODC↑	Rat embryo calvaria	[422]
	Kidney	ODC↑	Intact or HYPOX rat	[263,382]
	Brain; liver	ODC↑	Rat	[263]
	Testis; ovary	ODC↑	Rat	[263]
PTH	Chondrocytes	ODC↑; PA↑; c-SAT↑	Rabbit ribs	[234,438–442]
	Bone cells	ODC↑	Rat embryo calvaria	[422]
	Osteoclasts; osteoblast-like cells	ODC↑	Neonatal mouse calvaria	[25]
	Osteoblasts	ODC↑; PUT↑	Calvaria of fetal rat or chick embryo	[209,210,454, 454b]
	Periosteal fibroblast	ODC =	Chick embryo calvaria	[210]

TABLE 1 (continued)

Hormone or prohormone	Organ(s) and/or tissue(s) and/or cultured cells	Polyamine effect(s)	Remarks	Ref. No.
PTH	Kidney	ODC↑	HYPOX-PARATHYRX-THYRX rat	[382]
	3T3 or L or BHK cells	ODC =		[441]
EPI	Granulosa cells	ODC↑	Immature or mature pig	[458,459, 461,462]
	Bronchial epithelial cells	ODC↑	Human	[469]
	Liver	ODC =	HYPOX rat	[341]
	Hepatocytes	ODC =	Fetal rat	[364]
	Spleen	ODC↑	Rat	[429]
	Heart	ODC↑	Fetal mouse	[59,479]
	Perfused heart	ODC↑	Rat	[112]
	Lung	ODC↑	Rat	[176]
	Brain	ODC↑	Neonatal rat	[252]
	Testis; Leydig cells; seminiferous tubules	ODC↑	Immature rat	[219,221]
	Parotid explants	ODC↑; SAMDC↑	Rat	[144]
N-EPI	Granulosa cells	ODC↑	Immature pig	[458]
	Testis	ODC↓	Immature rat	[336]
	Testis; Leydig cells; seminiferous tubules	ODC↑	Immature rat	[219,223]
	Heart	ODC↑	Rat	[100]
	Perfused heart	ODC↑	Rat	[112]
	Heart cells	ODC↑	Neonatal rat	[451c]
	Spleen	ODC↑	Rat	[429]
	Lung	ODC↑	Rat	[176]
	Brain	ODC↑	Neonatal rat	[251,252]
	Perfused heart; perfused aorta	ODC↑; SAMDC↑; PUT↑; SPD =; SP =	Rabbit	[39,259]
	Embryo	SPD↑; SP↑	Chick	[36]
Histamine	Cerebellum; cerebral cortex	ODC↑	Neonatal rat	[251]
Cortisol	Liver	ODC↑; SAMDC↑; SPD =; SP =	Rat	[40,60,80,341]
		ODC↑	HYPOX or ADRX rat	[295,341,342]
	Regenerating liver	ODC =	Partially hepatectomized rat	[40]
	Perfused liver	ODC =	Rat	[151]
	Hepatocytes	ODC =	Fetal rat	[364]
	Kidney	ODC↑	Intact or HYPOX rat	[27,342,384]
		ODC↑	HYPOX-ADRX rat	[384]
		ODC =; SAMDC =	Intact or ORCHX mouse	[31,293]
	Thyroid	ODC =	Rat	[343]
	Brain	ODC↓	Neonatal rat	[7]
		ODC =	Neonatal rat	[357]
		ODC =	Mouse embryo; in vitro	[20]
	Brain cells	ODC =	Chick embryo	[297]
	Thymus	ODC↓; SAMDC↓	HYPOX-ADRX rat	[383]

TABLE 1 (continued)

Hormone or prohormone	Organ(s) and/or tissue(s) and/or cultured cells	Polyamine effect(s)	Remarks	Ref. No.
Cortisol	Duodenum	ODC↑; SAMDC↑	Rachitic chick	[428]
	Lung	ODC =	Rat	[176]
	Adrenals	ODC =	HYPOX rat	[197]
		ODC↓	HYPOX rat	[342]
	Mammary gland explants	ODC =; PA-Up =	Pregnant mouse	[163,277]
	Pelvic cartilage	ODC =	Chick embryo; in vitro	[32]
	Mesenchymal cells	ODC↓	Rat; in vivo	[331,332]
Cortisone	Liver (L); thymus (T)	ODC-L↑; ODC-T↓	Young rat	[340]
	Kidney	ODC↑	ADRX mouse	[6]
	Testis	ODC =; SAMDC =; SPD =	HYPOX rat	[216]
Cortico-sterone	Liver	ODC↑	ADRX rat	[62]
	Spinal cord	ODC↑	ADRX rat	[284]
	Thymus	ODC↓; SAMDC↓	HYPOX-ADRX rat	[383]
	Kidney (cortex and medulla)	ODC↑	Rat	[200]
	Hippocampus; cerebellum	ODC↑; SAMDC =; c-SAT↑	ADRX rat	[62,140]
	Telencephalon	PA =	Mouse	[450]
	Mesenchymal cells	ODC↓	Rat; in vivo	[331,332]
Aldosterone	Kidney	ODC↑	HYPOX or HYPOX-ADRX rat	[382]
	Spinal cord	ODC↑	ADRX rat	[284]
	Liver (L); brain (B)	ODC-L =; ODC-B↑	ADRX rat	[62]
	Thymus	ODC =; SAMDC =	HYPOX-ADRX rat	[383]
	Hippocampus; cerebellum	c-SAT =	ADRX rat	[140]
	Lung	ODC =	Rat	[176]
Vitamin D_3	Duodenum	ODC↑; SAMDC =; SP =; PUT↑; SPD↓; SP-B-Prot↑; c-SAT↑	Rachitic chick	[244,245,412–415,428,437]
		SAMDC↑	Rachitic chick	[428]
		ODC↑	Vit. D-deficient rat	[423]
	Liver	ODC =; PA =; c-SAT =	Rachitic chick	[412,413]
	Kidney; pancreas	ODC =; PA =; c-SAT↑	Rachitic chick	[412,413]
	Kidney	ODC =	HYPOX rat	[382]
	Bone; cartilage	ODC =; c-SAT =	Rachitic chick	[412,413]
	Chondrocytes; 3T3 cells	ODC =	Rabbit	[381,440]
	Epiphysis (E); diaphysis (D)	ODC-E =; ODC-D↑	Tibiae; vit. D-deficient rat	[423]
	Osteoblasts	ODC↓	Chick embryo calvaria	[209]
	Osteoblast-like cells	ODC =	Neonatal mouse calvaria	[25]
	Epidermis	ODC =	Mouse	[45]
	Monocytes	ODC-mRNA =; ODC↑	Human	[483b]

TABLE 1 (continued)

Hormone or prohormone	Organ(s) and/or tissue(s) and/or cultured cells	Polyamine effect(s)	Remarks	Ref. No.
Testosterone	Prostate (dorsal and/or ventral)	ODC↑; SAMDC↑; PA↑; SPD-SYN↑; SP-SYN↑; MTA↑; MTA-Pase↑; ODC-mRNA↑; SP-B-Prot↑	Intact or ORCHX rat	[23,65,89,89b, 99,165,166, 204,229,248, 255,270,302, 318,327,402, 416,443,470, 473,474]
	Prostate (dorsal (D) and/or ventral (V))	ODC↑; SAMDC↑	HYPOX rat	[302]
		ODC↑; SAMDC-D↑; SAMDC-V↓; ODC-mRNA↑; $t_{1/2}$ ODC =; SAMDC-mRNA↑	Adult or aged rat	[363,395, 407–409]
		ODC =; SAMDC-D↑; SAMDC-V =; $t_{1/2}$ SAMDC =	Juvenile rat	[395,407,408]
	Seminal vescicles	ODC↑; SAMDC↑; PA↑; SPD-SYN↑; SP-SYN↑; MTA↑; MTA-Pase↑	ORCHX rat	[65,89, 165,166, 255,318]
		ODC-mRNA↑		[23]
	Liver	ODC =	Intact or HYPOX or ADRX rat; mouse	[62,105, 295,341]
		SAMDC↓	OVX rat	[464]
		MTA-Pase =	ORCHX rat	[270]
	Lung	ODC↑	Rat	[176]
		MTA-Pase =	ORCHX rat	[270]
	Ovary; uterus	ODC =	Immature or mature rat	[168,175,282]
	Ovary	ODC↓	Chick embryo	[444]
	Testis	ODC =; SAMDC↑; SPD↑	HYPOX rat	[216]
	Sertoli cells	ODC-induction↓	Immature rat	[434]
		ODC-mRNA↓		[467b]
		ODC =	Bovine	[91]
	Epididymis	ODC-I =; ODC-O↑	Intact (I) or ORCHX (O) rat	[68,69]
	Brain	ODC =; c-SAT =	ADRX rat	[62,140]
	Kidney	ODC↑; SAMDC =/↑; PA↑	ORCHX mouse	[37,101,120,121, 293,310–312, 361]
		ODC↑; ODC-mRNA↑	ORCHX rat	[23,324]
		ODC↑	HYPOX mouse	[31]
	Kidney cortex	ODC↑; PA↑	Mouse; in vivo/in vitro	[177]
	Kidney	ODC↑; $t_{1/2}$ ODC↑; $t_{1/2}$ SAMDC↑; PA↑	Mouse	[18,31,105,145, 186,246,247, 300,301,396–398,452]
		ODCs↑	Mouse	[171,187,207, 309,315]
		ODC-mRNA(s)↑; ODC↑	Mouse	[17–19,123,158, 180,181,240, 241,338b, 405]

TABLE 1 (continued)

Hormone or prohormone	Organ(s) and/or tissue(s) and/or cultured cells	Polyamine effect(s)	Remarks	Ref. No.
Testosterone	Kidney	ODC =	Neonatal mouse	[300]
		ODC =	Tfm/Y mouse	[31,207,247]
	BHK cells	ODC↑		[206]
	Gastrocnemius; levator ani	ODC↑; PUT↑; SPD↑; SP =	ORCHX mouse or ORCHX rat	[74,314]
	Soleus	ODC =; PUT =; SPD↓; SP↓	ORCHX mouse or ORCHX rat	[314]
	Spinal cord	ODC =	ADRX rat	[284]
	Submaxillary gland	ODX =; SAMDC↑; SPD↑; SP↑	ORCHX rat	[141]
	Endochondral bone formation	ODC =	Rat; in vivo	[331]
	Heart	ODC =; PUT↑; SPD =; SP =	ORCHX mouse or ORCHX rat	[314]
	Myocytes; ventricular cubes	ODC↑; Pa↑	Rat	[178]
	Duodenum	ODC =; SAMDC =	Rachitic chick	[428]
	CHO cells	ODC↑		[61]
DHT	Kidney	ODC↑	Mouse; rat	[125,247]
		ODC↑	HYPOX mouse	[293]
		ODC =	Tfm/Y mouse	[247,293]
		ODC↑; SAMDC =	ORCHX mouse	[293]
	Prostate	ODC↑; PUT↑; SPD↑; SP =; SP-B-Prot↑; SP-B-Prot-mRNA↑	Intact or ORCHX rat	[41,124,243, 363,443]
Inhibin-like activity	Prostate (P); testis (T)	ODC-P↑; ODC-T =	Rat	[264]
Estradiol	Uterus	ODC↑; SAMDC↑; PUT =; SPD↑; SP =; ODC-mRNA↑; SPD-SYN↑; SP-SYN↑	Immature or mature rat	[28,165,168,169, 192,193,201, 202,282,358, 359,410,484]
		ODC↑	Neonatal rat	[282]
		ODC↑	HYPOX rat	[55]
		ODC↑; ODC-mRNA↑; PA↑; SAMDC↑; MTA-Pase↑	OVX rat	[28–30,55,194, 255,270,282, 330,368,369, 466,467]
		ODC↑; ODC-mRNA↑	OVX mouse	[43,44]
	Oviduct	ODC↑	Chick	[29,201,202]
	Ovary	ODC =	Mature or immature rat	[153,175]
		ODC↑	Chick embryo	[444]
		ODC↑	Pregnant rat	[122]
	CHO cells	ODC =		[61]
	Granulosa cells	ODC =	Pig	[457]
	Sertoli cells	ODC-induction =	Immature rat	[434]
	Liver	ODC↑/=; SAMDC↑; SPD↑; SP↑; SPD-SYN =; SP-SYN =	Immature chick	[79,258]
		ODC↓	Rat	[265]
		SAMDC =; MTA-Pase =	ORCHX rat	[270,464]
		ODC↑	HYPOX rat	[341]
		ODC =	ADRX rat	[62,295]

TABLE 1 (continued)

Hormone or prohormone	Organ(s) and/or tissue(s) and/or cultured cells	Polyamine effect(s)	Remarks	Ref. No.
Estradiol	Lung	ODC↑; MTA-Pase =	Rat or ORCHX rat	[176,270]
	Duodenum	ODC =; SAMDC =	Rachitic chick	[428]
	Spinal cord	ODC =	ADRX rat	[284]
	Kidney	ODC↑; SAMDC↑; PA↓	ORCHX hamster	[266]
		ODC↑; SAMDC↑/=; PA↑	ORCHX rat or OVX rat	[265,293,324]
		ODC =	Female or Tfm/Y mouse	[247]
	BHK cells	ODC↑		[206]
	Pituitary	ODC↑; PA↑	OVX or OVX-HYSTX rat; intact rat	[106,316,317]
	Brain	ODC =; c-SAT =; PA =	ADRX rat or OVX-HYSTX rat	[62,106,140]
	Levator ani	ODC↑	ORCHX rat	[74]
	Müllerian duct	ODC↑	Chick embryo	[444]
	Endochondral bone formation	ODC =	Rat	[331]
	Prostate	ODC =/↓; SAMDC =; PUT =; SPD =; SP-B-Prot =	Intact or ORCHX rat	[243,265, 363,416]
	Testis	ODC↓	Rat; chick embryo	[265,444]
	Thymus; adrenals	ODC↓	Rat	[265]
	Interstitial cells	ODC =	Rat testis	[290]
Progesterone	Ovary	ODC =	Immature or mature rat	[153,175]
	Uterus	ODC =	Neonatal or adult rat; pregnant hamster	[201,202, 213,282]
	Uterine epithelium	ODC↑	OVX mouse	[43]
	Endometrial fragments	SAMDC↑; PUT =; SPD↑; SP =	Guinea pig	[87]
	Spinal cord	ODC =	ADRX rat	[284]
	Liver; brain	ODC =	ADRX rat	[62]
	Kidney	ODC =; SAMDC =	ORCHX rat or ORCHX mouse; intact mouse	[31,265,293]
	BHK or L cells	ODC↑		[206]
	Mammary gland	SAMDC↓; SPD↓; SPD-SYN↓	Mouse; in vitro	[281]
	Brain	c-SAT =	ADRX rat	[140]
	Oviduct	ODC = (in vitro); ODC↑ (in vivo)	Immature chick	[29,201,202]
	Endochondral bone formation	ODC =	Rat	[331]
Relaxin	Uterus; pubic symphysis	ODC↑	Immature or mature or OVX mouse	[26]

amine) effects of hormones is sometimes still elusive.

It is, however, conceivable that the regulation of polyamine biosynthesis by hormones is a general, though neither unique nor sufficient, mechanism whereby the typical effects induced by

hormones are achieved. In other words, hormones may involve the polyamine biosynthetic pathway to procure their major biochemical and biological effects. Mammalian hormones are able to modulate (although to a variable extent) the synthesis of nucleic acids and proteins in responsive tissues (Martin, 1985; Norman and Litwack, 1987; Cahill, 1989); polyamines are similarly intimately involved with nucleic acid and protein synthesis. Hormones that stimulate growth and/or differentiation of mammalian cells also stimulate polyamine biosynthesis in their target organs and/or tissues; hormones that stop growth and/or differentiation in their target tissues reduce or abolish polyamine biosynthesis in the same tissues. Some hormones stimulate polyamine biosynthesis in one tissue and inhibit it in another; the same is true for hormones, which are anabolic for some organs and catabolic for others. Lastly, Table 1 also shows that there are some instances of conflicting reports on the polyamine effect(s) of a given hormone for a particular tissue. This may reflect different experimental conditions.

Finally, the possibility that polyamines, especially spermidine and spermine, may act as modulators of hormone action at the cell membrane level has been recently canvassed. It has been demonstrated that these polyamines enhance the binding of several hormones, such as GH, FSH and insulin, to the corresponding membrane receptor (D'Abronzo et al., 1985; Swift and Dias, 1986; Pedersen et al., 1989). Furthermore, it has been demonstrated that natural polyamines enhance the stability of estrogen-receptor complexes (Thomas and Kiang, 1987). Thus, the fate of the hormone-receptor binding may be influenced by the surrounding polyamine environment.

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Review

Polyamines and mammalian hormones

Part II: Paracrine signals and intracellular regulators

Giuseppe Scalabrino and Erna C. Lorenzini

Institute of General Pathology and C.N.R. Center for Research in Cell Pathology, University of Milan, 20133 Milan, Italy

Key words: Polyamines; Mammalian hormones; Paracrine signal; Intracellular regulator

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Three passions, simple but overwhelmingly strong, have governed my life: the longing for love, the search for knowledge, and unbearable pity for the suffering of mankind.

B. RUSSELL, *Autobiography*, Prologue

1. Parahormones and growth factors regulating content, biosynthesis and interconversion of polyamines in mammalian organs, tissues and cultured cell lines

Mammalian parahormones and growth factors with effects on the biosynthesis and/or interconversion of polyamines in different mammalian systems are listed in Table 1.

Address for correspondence: G. Scalabrino, MD, Istituto di Patologia Generale, Università degli Studi di Milano, Via Mangiagalli 31, 20133 Milan, Italy.

1.1. Discussion of Table 1

There are a number of comments that are specific for the data in this table, and at the same time complementary to those in Table 1 in the first part of this review (Scalabrino et al., 1991). First, both cAMP and cGMP have been shown to induce both PBD activities in mammalian tissues. This phenomenon can be primary, i.e., a response to either of these cyclic nucleotides per se (see Table 1), or secondary, i.e., with cAMP as a second messenger for hormones (see Table 1 in part I of this review). Other data supporting the view that PBD enzyme induction is mediated by cyclic nucleotides are the lack of induction by a stimulat-

ing hormone in variants of mammalian cell lines that are deficient in some steps of cAMP metabolism (including cAMP-dependent protein kinases) (Kudlow et al., 1980; Honeysett and Insel, 1981; Van Buskirk et al., 1985).

Second, it appears from Table 1 that most prostaglandins, notably PGE and PGI_2, induce ODC activity in different types of mammalian cell lines. Both these prostaglandins are known to have cAMP as their second messenger (Rao, 1988). In contrast, though $PGF_{2\alpha}$ has also been shown to induce ODC activity, it does not use cAMP as second messenger (Rao, 1988). Again, as mentioned in the discussion of Table 1 in part I, hormones such as steroids that do not require cAMP as mediator for their biological effects also induce PBD activities in different tissues. It is therefore abundantly clear that ODC induction in mammalian cells is mediated in some instances via cyclic nucleotides (especially cAMP) and in other instances is independent of cAMP.

Third, studies with mutants of different receptor-defective cell lines have shed further light on the specific nature of stimulation of polyamine

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Abbreviations:
ADRX = adrenalectomized animals
BHK = baby hamster kidney
CAM = calmodulin
cAMP = adenosine 3,5-cyclic monophosphate
CCK = cholecystokinin
CHO = Chinese hamster ovary
cGMP = guanosine 3,5-cyclic monophosphate
ConA = concanavalin A
CTLL = cytotoxic limphoid line
DFMO = α-difluoromethylornithine
DG = 1,2-diacylglycerol
EGF = epidermal growth factor
ER = endoplasmic reticulum
ES = estrogen
FGF = fibroblast growth factor
GM-CSF = colony-stimulating factor for granulocytes and macrophages
G_p = guanine nucleotide-regulatory binding protein
HYPOX-ADRX = hypophysectomized-adrenalectomized animals
HYPOX = hypophysectomized animals
IF = interferon
IL = interleukin
IP = inositol 4-monophosphate
IP_2 = inositol 1,4-bisphosphate
IP_3 = inositol 1,4,5-trisphosphate
IP_4 = inositol 1,3,4,5-tetraphosphate
IP_5 = inositol 1,3,4,5,6-pentaphosphate
IP_6 = inositol hexaphosphate
LH = luteinizing hormone
MCSF-1 = macrophage colony-stimulating factor 1
MG = monoacylglycerol
MGBG = methylglyoxal bis-(guanylhydrazone)
MSA (IGF-II) = multiplication stimulating activity (insulin-like growth factor II)
α-MSH = melanocyte-stimulating hormone
MTA = 5′-deoxy-5′-methylthioadenosine
NGF = nerve growth factor
NSILA = non-suppressible insulin-like activity
ODC = L-ornithine decarboxylase (EC 4.1.1.17)
ORCHX = orchiectomized animals
PA = phosphatidic acid
PBD = polyamine biosynthetic decarboxylases
PDGF = platelet-derived growth factor
PG = pentagastrin
PGE_1 = prostaglandin E_1
PGE_2 = prostaglandin E_2
$PGF_{1\alpha}$ = prostaglandin $F_{1\alpha}$
$PGF_{2\alpha}$ = prostaglandin $F_{2\alpha}$
PGI_2 = prostaglandin I_2
PHA = phytohemagglutinin
PI = phosphatidylinositol
PIP = phosphatidylinositol 4-phosphate
PIP_2 = phosphatidylinositol 4,5-bisphosphate
PRL = prolactin
PTH = parathormone
PUT = putrescine
PUT-Up = putrescine uptake
R = receptor
SAMDC = *S*-adenosyl-L-methionine decarboxylase (EC 4.1.1.50)
SAT = spermidine/spermine acetyltransferase
SGF = sarcoma growth factor
SP = spermine
SPD = spermidine
SP-Up = spermine uptake
SPD-Up = spermidine uptake
SUBMX = submandibulectomized animals
T_3 = triiodothyronine
T_4 = thyroxine
TGF = transforming growth factor
TNF = tumor necrosis factor
TS = testosterone

Symbols:
= No variation in comparison with controls
↑ Increase in comparison with controls
↓ Decrease in comparison with controls

TABLE 1

EFFECTS OF MAMMALIAN PARAHORMONES AND MAMMALIAN FACTORS (BOTH NORMAL AND NEOPLASTIC) ON CONTENT AND/OR BIOSYNTHESIS AND/OR INTERCONVERSION OF POLYAMINES OF MAMMALIAN ORGANS, TISSUES AND CULTURED CELL LINES

Parahormone or growth factor	Organ(s) and/or tissue(s) and/or cultured cells	Polyamine effect(s)	Remarks	Ref. No.
PGE_1	Heart	ODC =	Rat	[112]
	Liver	ODC ↑	Neonatal or adult rat	[107,108,112]
	Hepatocytes	ODC =	Rat	[236]
		ODC ↑	Fetal rat	[196]
	Mammary explants	ODC ↑	Pregnant mouse	[161,255]
	Granulosa cells	ODC ↑	Porcine follicles	[163]
	Corpora lutea	ODC =	Pregnant cow	[121]
	CHO cells	ODC ↑ (+ asparagine)		[29]
	Thyroid slices	ODC ↑	Dog	[148]
	Osteoblasts	ODC ↑	Chick embryo calvaria	[126]
	Pelvic cartilage	ODC ↑	Chick embryo	[20]
PGE_2	Thyroid gland	ODC =	Rat	[185]
	Adrenals	ODC ↑	Rat	[185]
	Testis; Leydig cells; seminiferous tubules	ODC ↑	Immature rat	[134,135,137]
	Testis; interstitial cells	ODC =	Rat; in vitro	[167]
	Ovary	ODC ↑	Adult or pregnant rat	[78,111]
	Uterus	ODC =	Prepubertal rat	[266]
	Granulosa cells	ODC ↑	Immature or mature pig	[163,166,243, 244,246–248]
	Mesenchymal cells	ODC =	Mouse embryo	[177]
	Mammary explants	ODC ↑	Pregnant mouse	[255]
		ODC =	Pregnant mouse	[161]
	Bone cells	ODC ↑	Rat embryo calvaria	[220]
	Macrophages	ODC ↑	Peritoneum; guinea pig	[178]
	Osteoblast-like cells	ODC ↑	Mouse calvaria	[16]
$PGF_{1\alpha}$	Thyroid slices	ODC ↓	Dog	[148]
$PGF_{2\alpha}$	Uterus	ODC ↓	Pregnant hamster	[131]
	Corpora lutea	ODC ↑	Pregnant rat	[78]
		ODC =	Pregnant ox	[121]
	Testis; Leydig cells; seminiferous tubules	ODC ↑	Immature rat	[134–136,138]
	Mammary explants	ODC ↑	Pregnant mouse	[255]
	Granulosa cells	ODC =	Porcine follicles	[163]
	BHK cells	ODC =		[122]
	Mesenchymal cells	ODC =	Mouse embryo	[177]
PGI_2	Mammary explants	ODC ↑	Pregnant mouse	[255]
NSILA	Brain cells	ODC ↑	Fetal rat	[261]
	Fibroblasts	ODC ↑	Chick embryo	[74]
MSA (IGF-II)	Granulosa cells	ODC ↑	Pig	[241,248]
	Fibroblasts	ODC ↑; PUT-Up ↑	Chick embryo	[219]
	L6 myoblasts	ODC ↑	Rat	[46]
	Soleus	ODC ↑	Diabetic rat; in vitro	[27,28]
	Pelvic cartilage	ODC ↑	Chick embryo	[20]
	3T3 cells	ODC ↑		[160]
	Intestinal epithelial cells	ODC ↑		[63d]
Neuromedin C	Pancreas	PUT = ; SPD = ; SP =	Rat	[207]

TABLE 1 (continued)

Parahormone or growth factor	Organ(s) and/or tissue(s) and/or cultured cells	Polyamine effect(s)	Remarks	Ref. No.
cAMP (or derivatives)	Liver	ODC-I↑; ODC-H =; ODC-A↑; ODC-HA =	Intact (I) or HYPOX (H) or ADRX (A) or HYPOX-ADRX (HA) rat	[37,45,81,112, 117,118,184]
	Kidney; adrenals	ODC-H↑; ODC-HA =	HYPOX (H) or HYPOX-ADRX (HA) rat	[184]
	Adrenals	ODC↓	Rat; in vitro	[184]
	Liver	ODC↑	Neonatal rat	[45,107]
	Perfused liver	ODC↓	Rat	[117,118]
	Brain	ODC↑	Neonatal rat	[107]
	Hepatocytes	ODC↑	Adult or fetal rat	[128,196]
		ODC↓	Rat	[236]
	Heart	ODC =	Rat	[112]
	Perfused heart	ODC↑	Rat	[2]
	Thyroid	ODC =	Rat	[185,267]
		ODC↑	Rat; mouse; in vitro	[61,62,206]
	Thyroid slices	ODC↑	Dog	[148]
	Cerebellum	ODC↑	Neonatal rat	[150]
	Brain cells	ODC =	Chick embryo	[173]
	Epidermis	ODC =	Mouse	[176]
	Epidermal cells	ODC =	Chick embryo	[223]
	Testis; Leydig cells; seminiferous tubules	ODC↑	Mature or immature rat; in vitro	[135,137,138, 167,181]
	Testis	ODC =	Neonatal mouse	[224]
	Sertoli cells	ODC↑; SPD↑; SP↑	Mature or immature rat	[228,229]
		ODC↓	Rat	[139]
	Prostate	ODC =; SAMDC =	ORCHX rat	[142]
	Corpora lutea	ODC =	Pregnant ox	[121]
	Ovarian cells	ODC↑	Pubertal rat	[99]
	Granulosa cells	ODC↑	Immature or mature pig	[163,164,166, 241,243, 244–246]
	CHO cells	ODC↑ (+ amino acid)		[29–31]
	CHO mutant cells	ODC↑; PUT↑		[237]
	Mammary explants	ODC↑	Mid-pregnant mouse	[1,161]
		ODC = /↑	Pregnant mouse	[256]
	Costal chondrocytes	ODC↑; cSAT↑	Rabbit	[145,232,233]
	Pelvic cartilage	ODC↑; $t_{1/2}$ ODC↓	Chick embryo; in vitro	[20]
	Osteoblasts	ODC↑	Calvaria of chick embryo	[126,127]
	Osteoblast-like cells	ODC↑	Mouse calvaria	[16]
	Bronchial epithelial cells	ODC↑	Human	[254]
	3T3 cells	ODC =		[190]
	Swiss 3T3 cells	ODC↑ (+ asparagine)		[113]
	L cells	ODC↑	Mouse	[260]
	Parotid explants	ODC↑; SAMDC↑	Rat	[89]

TABLE 1 (continued)

Parahormone or growth factor	Organ(s) and/or tissue(s) and/or cultured cells	Polyamine effect(s)	Remarks	Ref. No.
cAMP (or derivatives)	BHK cells	ODC↑		[122]
	Adrenocortical cells	PUT-Up =	Bovine	[51]
	Cultured superior cervical ganglia	ODC↑	Young rat	[132]
	Lymphocytes	ODC =	Human	[103]
		cSAT↑	Ox	[146]
	Keratinocytes	ODC↑	Neonatal mouse	[194]
	CT6 cells	ODC =; ODC-mRNA↓	Mouse	[49]
	NSF-60.8 cells	ODC =; ODC-mRNA =	Mouse	[50]
cGMP (or derivatives)	Epidermis	ODC =	Mouse	[176]
	Mammary explants	ODC =	Pregnant mouse	[256]
	Costal chondrocytes	ODC =	Rabbit	[232]
	Parotid explants	ODC =; SAMDC =	Mouse	[89]
	Sertoli cells	ODC =	Rat	[139]
	Hepatocytes	ODC↑	Rat	[236]
	Adrenocortical cells	PUT-Up =	Bovine	[51]
	Perfused liver	ODC↑	Rat	[117]
EGF	Stomach; duodenum	ODC =	Rat	[209]
		ODC↑; PUT↑	Neonatal or adult mouse	[54,102]
	Ileum	ODC↑	Rat	[209]
	Colon	ODC =	Rat; neonatal mouse	[54,209]
	Colonic mucosal explants	ODC↑	Rat	[2b]
	Duodenal or ileal mucosa; intestine; enterocytes	ODC↑; SAMDC↑; immunoreactive ODC↑	Rat; mouse; spf/Y mouse	[57,95, 141,155]
	Thymus; spleen	ODC =	Rat; neonatal mouse	[197,198,224]
	Testis; interstitial cells	ODC =	Rat; in vitro	[167]
	Testis	ODC↑; PUT↑; SPD =; SP =	Neonatal mouse	[155,224]
	Sertoli-spermatogenic cells; seminiferous peritubular cells	ODC↑	Immature rat; in vitro	[218]
	Skin	ODC↑	Neonatal or adult mouse	[14,34,54,223]
	Epidermal cells	ODC↑; PUT↑; SPD↑; SP =	Chick embryo	[176]
	Keratinocytes	ODC↑	Bovine	[58]
	Liver	ODC↑	Neonatal mouse	[155,224]
	Hepatocytes	ODC↑	Rat	[236]
	Kidney	ODC↑	Neonatal mouse	[155,224]
		ODC =	Rat	[197]
	Kidney cells	ODC↑	Rat	[172]
	Brain	ODC =	Neonatal mouse	[224]
		ODC↑	Immature mouse	[155]
	Heart	ODC =	Neonatal mouse	[54]
		ODC↑	Immature mouse	[155]

TABLE 1 (continued)

Parahormone or growth factor	Organ(s) and/or tissue(s) and/or cultured cells	Polyamine effect(s)	Remarks	Ref. No.
EGF	Parotid explants	ODC =; SAMDC =; PUT =; SPD =; SP =	Rat or SUBMX mouse	[90]
	Fibroblasts	ODC↑	Rat embryo	[252,253]
		ODC↑; PUT-Up↑; SPD-Up↑; SP-Up↑; ODC-mRNA↑	Human; mouse	[34,92,97]
	Mesenchymal cells	ODC↑; PUT-Up↑	Mouse embryo	[63,177]
	Mammary explants	ODC↑	Pregnant mouse	[1]
	Swiss 3T3 cells	ODC↑	Mouse	[222]
	3T3 cells	ODC =		[58,84]
	Granulosa cells	ODC↑	Immature or mature pig	[165,241,243]
	Bronchial epithelial cells	ODC↑	Human	[254]
	Macrophages	PUT-Up =	Mouse	[34]
	3T3 (transfected lines)	ODC-mRNA↑; ODC↑		[214b]
	Epithelial cells	PUT-Up↑	Pig kidney	[173b]
	Cultured pancreatic islets	SPD =; SP =	Fetal rat	[214c]
FGF	Granulosa cells	ODC =	Mature or immature pig	[165,243]
	Swiss 3T3 cells	ODC↑	Mouse	[222]
	3T3 fibroblasts	ODC↑	Mouse	[84]
	BALB/c 3T3 cells	ODC-mRNA↑		[100]
PDGF	Smooth muscle cells	ODC↑; PUT↑; SPD =; SP =	Rat; aorta	[235]
	3T3 cells	ODC↑		[84,249]
		ODC =		[113,189]
	Sertoli-spermatogenic cells (SC); seminiferous peritubular cells (SPC)	ODC-SC =; ODC-SPC↑	Immature rat; in vitro	[218]
	3T3 fibroblasts	ODC↑ (+asparagine)		[189]
	BALB/c 3T3 cells	ODC-mRNA↑		[36,100]
	Intestinal epithelial cells	ODC↑		[63c]
NGF (2.5S or 7S)	Brain; adrenals	ODC↑	Intact or HYPOX or ADRX rat	[3,87,88,119, 153,191]
	Brain	ODC =	Neonatal rat	[68]
	Liver	ODC↑; ODC-A =; ODC-N =	Intact or HYPOX or ADRX (A) or neonatal (N) rat	[3,68,88,153]
	Kidney	ODC-I↑; ODC =	Intact (I) or HYPOX or ADRX rat	[153]
	Superior cervical ganglia	ODC↑	Neonatal rat	[76]
	Superior cervical ganglia	ODC↑	Young rat; in vitro/in vivo	[68,103]
	Fibroblasts	ODC =	Chick embryo	[74]
IL-1	Natural killer cell-like (NK); helper T-cell line (HT)	ODC-NK↑ (+ConA); ODC-HT↑ (+ConA)	Human (NK); mouse (HT)	[43]

TABLE 1 (continued)

Parahormone or growth factor	Organ(s) and/or tissue(s) and/or cultured cells	Polyamine effect(s)	Remarks	Ref. No.
IL-1	Mononuclear leucocytes	ODC↑ (+PHA)	Peripheral blood	[55]
	Spleen; liver	ODC↑	Mouse	[42,44]
	T lymphocytes	ODC↑ (+PHA)	Human blood	[56]
	Spleen (S); liver (L); brain (B); thymus (T); heart (H)	ODC-L↑; ODC-H↑; ODC-S=; ODC-T=; ODC-B=	Mouse	[40]
IL-2	Mononuclear leucocytes	ODC↑ (+PHA)	Peripheral blood	[55]
	CTLL cells	ODC↑; ODC-mRNA↑; PUT↑; SPD↑; SP=		[17,114, 115,264]
	Mononuclear cells	PUT↑; SPD↑; SP↑	Rat spleen	[264]
	T lymphocytes	ODC↑ (+PHA)	Human blood	[56]
	Thymocytes	ODC↑	Rat	[18]
	CT6 cells; CTB6 cells	ODC↑; ODC-mRNA↑		[49]
	FDC-P1 cells	ODC↑	Mouse	[50]
IL-3	FDC-P1 cells	ODC↑; PUT↑; SPD↑; SP=		[17,50]
	CT6 cells	ODC=		[49]
IF	Swiss 3T3 cells	ODC-induction↓	Mouse	[221,222]
IF ($\alpha+\beta$)	BALB/c3T3 cells	ODC-induction↓	Mouse	[36]
	3T3-L1 cells	PUT=; SPD=; SP=		[234]
	Regenerating liver	PUT↓; SPD=; ODC (first peak)↓	Mouse	[156,157]
	Liver; spleen	ODC=	Mouse	[41]
IF (γ)	NSF-60.8 cells	GM-CSF-induced ODC↓; GM-CSF-induced ODC-mRNA↓	Mouse	[50]
	Spleen	ODC↑	Mouse	[44]
Methionine-enkephalin	Brain cells	ODC=	Fetal rat	[261,262]
Enkephalin	Liver (L); brain (B)	ODC-L↑; ODC-B=	Rat	[88]
α-Endorphin	Lung	ODC↑	Rat	[104]
β-Endorphin	Kidney	ODC↑	Intact or HYPOX rat	[72]
	Pancreas; adrenals; small intestine; thymus; heart; spleen; lung; liver; gut	ODC=	Intact or HYPOX rat	[72]
	Brain (B); heart (H); kidney (K); liver (L)	ODC-B-N↑; ODC-H-N↑; ODC-K-N=; ODC-L-N↑; ODC-Y=; ODC-B-A↑	Neonatal (N) or young (Y) or adult (A) rat	[5,6]
	Hippocampus	ODC↑ (+asparagine)	Rat; in vitro	[7]
TGF (α)	Brain; heart; liver; kidney; intestine; testis	ODC↑	Immature mouse	[155]
	Cultured pancreatic islets	SPD=; SP=	Fetal rat	[214c]
TGF (β)	Sertoli-spermatogenic cells; seminiferous peritubular cells	ODC↑	Immature rat; in vitro	[218]
SGF	Fibroblasts	ODC↑	Rat kidney	[172]
TNF	Liver; spleen	ODC↑	Mouse	[44]

biosynthesis by hormones and/or growth factors. For example, ODC levels were completely unaffected by estrogen in estrogen receptor-negative human breast cell lines (Lima and Shiu, 1985; Shiu et al., 1986). Similarly, a mutant PC12 cell line, which lacks the receptor for NGF, is unresponsive to NGF in terms of ODC induction (Reinhold and Neet, 1989). Inhibition of ODC activity by glucocorticoids was not observed in a variant S49 lymphoma cell line which was resistant to glucocorticoids (Insel and Honeysett, 1981). In this regard it seems important to note that ODC induction by hormones is greatly reduced or absent in some receptor-positive neoplastic cells (especially in fully developed solid neoplasms) (Scalabrino et al., 1978; Frazier and Costlow, 1982; Shukla and Singhal, 1985; Suzuki et al., 1986; Glikman et al., 1990). However, this is not the case for all neoplastic cells, since prolactin stimulates ODC activity, and in some instances SAMDC, in rat Nb2 node lymphoma cells, a line absolutely dependent upon prolactin for mitogenesis (Richards et al., 1982; Buckley et al., 1986; Elsholtz et al., 1986; Russell et al., 1987). Similar effects have been found for parathyroid hormone in an osteogenic sarcoma cell line (van Leeuwen et al., 1988), for α-MSH in a mouse melanoma cell line (Scott et al., 1982; Frasier-Scott et al., 1983), for insulin and 17β-estradiol in a human breast cancer cell line (Hoggard and Green, 1986; Kendra and Katzenellenbogen, 1987) and for insulin in a hepatoma cell line (Goodman et al., 1988). Furthermore, ODC mRNA levels in hormone-unresponsive breast cancer cell lines have been found to be much higher than those in hormone-responsive breast cancer cell lines (Thomas, 1989), without, however, any significant difference in ODC activity levels between these two types of neoplastic cells (Thomas, 1989).

Fourth, there is clearly a two-way connection between polyamine and cyclic nucleotide synthesis in mammalian cells. To summarize the current state of knowledge, the three chief polyamines reduce cellular cAMP content by inhibiting cyclase activity (Khan et al., 1990b) and increasing phosphodiesterase activity, and at the same time increase cellular cGMP content by increasing guanylate cyclase activity and reducing cGMP-phosphodiesterase activity (Clo et al., 1983, 1988). In spite of the relatively few studies on this topic, this concomitant modulation of both cellular cAMP and cGMP content by polyamines appears to represent another mechanism by which polyamines regulate cell growth, since modifications of the intracellular cAMP/cGMP ratio clearly affect cell growth and replication. Therefore, a key regulatory loop may exist between those hormones acting through the cAMP pathway, cyclic nucleotides and polyamines. Such hormones enhance intracellular cAMP levels; cAMP in turn enhances intracellular ODC activity and thus intracellular polyamine content, causing a decrease in cAMP content and an increase in the cGMP level. This shifting of the intracellular cAMP/cGMP ratio by polyamines may have two effects, to reduce the biological effects of the hormonal action and to stimulate cell growth and replication (both due to the fall in cAMP content).

Finally, the few available studies have demonstrated that ODC induction is quantitatively reduced in cultured 3T3 fibroblasts and hepatoma cells made deficient in protein kinase C by pretreatment with phorbol 12-myristate 13-acetate (Hovis et al., 1986; Clo et al., 1988). Thus, as for the role of cyclic nucleotides in ODC induction, it appears that ODC induction can occur by activation of both a protein kinase C-dependent pathway and a protein kinase C-independent pathway (Jetten et al., 1985; Buckley et al., 1986; Hovis et al., 1986; Blackshear et al., 1987; Russell et al., 1987; Reinhold and Neet, 1989; van Leeuwen et al., 1989; Ghigo et al., 1990) (see also Section 2 of this part).

The studies performed in the last decade on growth factors as regulators of polyamine biosynthesis in different cell lines have both reinforced the concept that polyamines are intimately connected with cell growth and differentiation, and established the connections between ODC gene expression and cellular proto-oncogenes involved in cell growth and differentiation (vide infra). It is widely accepted that the term 'growth factor' includes not only peptides that affect cell replication, but also those that induce cell differentiation or enlargement without mitosis. The steps in the pathway whereby growth factors induce their cellular effects are very similar to that for traditional hormones and include highly specific

growth factor receptors and the second messenger system(s). Therefore, growth factors may be conveniently considered as a subclass of polypeptide hormones (Guroff, 1983–1985; Hopkins and Hughes, 1985; Russell and Van Wyk, 1989; Waterfield, 1989).

Like classic hormones, growth factors use only few classes of second messenger. One is cyclic nucleotides, especially cAMP (as demonstrated for NGF and EGF (Guroff et al., 1981; La Corbiere and Schubert, 1981)); there is, however, one report reporting no involvement of cAMP in ODC induction by NGF (Van Buskirk et al., 1985). An other class is the hydrolytic products of phosphatidylinositol (as for PDGF and the lymphocyte mitogens (Guroff, 1983–1985; Hopkins and Hughes, 1985; Russell and Van Wyk, 1989; Waterfield, 1989)).

There is a striking parallelism between traditional hormones and growth factors in terms of ODC induction in different mammalian cultured neoplastic cell lines. Like classical endocrine signals, both EGF and NGF induce ODC activity in the rat pheochromocytoma cell line PC12 (Guroff et al., 1981; La Corbiere and Schubert, 1981; End et al., 1982; Guroff and Dickens, 1983; Feinstein et al., 1985). Interestingly, it has been recently demonstrated that PC12 cells contain at least three distinct loci containing ODC sequences (ODC 1, ODC 2, ODC 3), but only ODC 1 is transcriptionally active and NGF-inducible (Muller et al., 1990). EGF also induces ODC activity in a human hepatoma cell line (Moriarity et al., 1981) and in a human epidermoid carcinoma cell line (Yazigi et al., 1989), but not in RSV-transformed rat fibroblasts (Widelitz et al., 1981) or in KB cells (Di Pasquale et al., 1978). Moreover, growth factors that have a general antiproliferative effect decreased ODC activity, as does IL-1 in a human melanoma cell line and human mammary cell lines (Endo et al., 1988) and γ-interferon in a human breast cancer cell line (Marth et al., 1989). The general conclusion can therefore be drawn that the growth factors have effects in terms of ODC induction in neoplastic cells similar to those of traditional hormones, being active in some instances and ineffective in others.

Some studies have quite clearly demonstrated that ODC induction is one important component in the complicated cascade of biochemical responses induced in mammalian cells (normal or neoplastic) by growth factors. Parenthetically, the distinction between normal and neoplastic growth factors has become debatable, since growth factors first identified as products of neoplastic cell lines were later found to also be produced by normal cells (Burgess, 1989; Hsuan, 1989). The cascade of responses includes the rapid and sequential activation of a set of genes (Kaczmarek, 1986; Rollins and Stiles, 1988; Kaczmarek and Kaminska, 1989; Woodgett, 1989). Briefly, studies on 3T3 cells stimulated by PDGF and PC12 cells stimulated by NGF have demonstrated that induction of the cellular proto-oncogene c-*fos* and c-*myc*, and of other genes such as ODC, β-actin and vimentin, is part of a general transcriptional response of the cells to growth factors (Greenberg et al., 1985, 1986).

It is clearly beyond the aim of the present review to discuss the mechanisms of action of the different growth factors in detail; we wish, rather, to emphasize a number of crucial points, as follows:

(a) The time course of activation of these genes varies considerably, since c-*fos* and β-actin genes are activated within a few minutes of the interaction between growth factor and its cell receptor (Greenberg et al., 1985, 1986; Kaczmarek, 1986; Rollins and Stiles, 1988; Kaczmarek and Kaminska, 1989; Woodgett, 1989). In contrast, c-*myc* expression is maximal approximately 1 h after stimulation by the growth factor, with ODC peaking a further hour later (Greenberg et al., 1985, 1986; Kaczmarek, 1986; Rollins and Stiles, 1988; Kaczmarek and Kaminska, 1989; Woodgett, 1989).

(b) The c-*fos*, c-*myc* and ODC genes are also activated during cell differentiation induced by various experimental means (Kaczmarek, 1986; Rollins and Stiles, 1988; Kaczmarek and Kaminska, 1989; Woodgett, 1989).

(c) Growth factors and lymphocyte mitogens control c-*fos* expression mainly at the level of gene transcription and c-*myc* expression at both the transcriptional and posttranscriptional levels (Rollins and Stiles, 1988). This indicates a clear parallelism with the similar regulation of ODC gene expression discussed in detail in part I of this review.

(d) The expression of c-*myc* and ODC genes shows similar changes in patterns of expression during the differentiation of an erythroleukemia cell line induced by chemical treatment, which thus does not entail interaction with specific cell receptors (Watanabe et al., 1986).

(e) The expression of PBD genes and the c-*myc* gene has been shown to have a similar kinetic course in 3T3 cells (Stimac and Morris, 1987), and 17β-estradiol has been shown to increase both c-*H-ras* and ODC gene expression at the peak of DNA synthesis (Cheng and Pollard, 1986).

(f) Growth factors are able to elicit other cellular modifications, some of which occur even earlier than the nuclear events (Rozengurt, 1986). One of the most important of these very rapidly observable responses is the activation of Na^+ influx into the cell, causing cytoplasmic alkalinization. In this regard, a very recent report demonstrates that when the different mechanisms of Na^+ transport are blocked by appropriate drugs the expression of c-*fos*, c-*myc* and ODC genes by a peculiar stimulating factor such as serum is not inhibited, although the concurrent induction of ODC activity is almost completely inhibited (Panet et al., 1989). Similarly, GM-CSF-evoked ODC induction and DNA synthesis were blocked in a human leukemic cell line in the presence of a specific inhibitor of the Na^+/K^+ exchanger (Ghigo et al., 1990).

(g) In recent years a variety of evidence has been presented to show that tyrosine kinases, which catalyze phosphorylation of tyrosine residues in proteins, play an important role in the regulation of cell growth and differentiation. The observation that several oncogene products as well as receptors for several growth factors (like EGF, PDGF, and MCSF-1) possess tyrosine kinase activity suggests that processes of cell growth and differentiation are associated with alteration in phosphotyrosine content (Gill, 1989; Hunter, 1989). In this regard, there are at least three important points to be made: (a) gastrin initially induces tyrosine kinase activity, and thereafter ODC activity, in rat colonic mucosa (Majumdar, 1990); (b) EGF increases ODC activity in 3T3 cells only if and when the *neu* tyrosine kinase is activated (Sistonen et al., 1989); (c) polyamines have been demonstrated to stimulate cytosolic protein tyrosine kinase from different sources (Sakai et al., 1988; Khan et al., 1990a).

As for polyamine uptake, it is important to note that hormones and growth factors regulate polyamine transport rate (see Table 1, and Table 1 in part I). It has, however, recently been demonstrated that transfection of rat fibroblasts with *ras* oncogene leads to increase of PBD and c-SAT activities, without affecting polyamine transport rate (Chang et al., 1988). In contrast, N-*myc* transfection of the same type of cells enhances polyamine uptake without affecting their biosynthesis (Chang et al., 1988). This clearly shows that polyamine biosynthesis and polyamine uptake are regulated by different genes.

Generally speaking, the links between the classical growth factors and the regulation of polyamine biosynthesis can also be considered to hold for the interleukins (cytokines), the relatively newly identified immuno-modulatory hormones (Harrison and Campbell, 1988; Strober and James, 1988), although the studies on this topic are — as one can see in Table 1 — very few.

2. A new perspective: polyamines and phosphoinositide metabolism

Hitherto we have dealt with the involvement of polyamines in the classical biochemical mechanisms of action of traditional hormones and growth factors. Until recently, phospholipids have been viewed mostly in the cell economy as structural substances, making up the membranes of mammalian cells and of their organelles and supporting protein molecules such as membrane-bound enzymes and receptors. This concept, however, is clearly outdated from the point of view of contemporary biochemistry. Indeed, protein hormones, neurotransmitters and growth factors have now been shown to provoke rapid and lasting changes in phospholipid metabolism. Moreover, phospholipids function as intracellular mediators for transducing the various effects of hormones, neurotransmitters, growth factors and tumor promoters in their target tissues.

Those hormones and neurotransmitters that use calcium as a second messenger specifically hydrolyse membrane phosphoinositides. Three mes-

senger molecules are produced as a result of phosphoinositide metabolism: 1,2-diacylglycerol (DG), inositol-1,4,5-triphosphate (IP_3) and arachidonic acid. The primary function of IP_3 is to rapidly mobilize calcium from intracellular, non-mitochondrial stores, mainly from the endoplasmic reticulum of the cell, while DG acts by stimulating protein kinase C-mediated phosphorylation. Together these two branches of this bifurcating pathway serve as an exceptionally versatile signaling mechanism that can control many short-term cellular response and long-term responses such as cell growth.

It is clearly beyond the aims of the present review to go in depth into the different biochemical aspects of the phosphoinositide signal pathway. The reader is referred to more ample reviews (Farese, 1983, 1984; Berridge, 1984, 1987a, b; Berridge and Irvine, 1984; Majerus et al., 1984; Nishizuka, 1984, 1986; Macara, 1985; Helmreich, 1986; Linden and Delahunty, 1989; Nahorski and Potter, 1989; Pelech and Vance, 1989; Shears, 1989; Downes and Macphee, 1990; Rana and Hokin, 1990).

Evidence for a close link between phosphatidylinositol metabolism and the control of cell

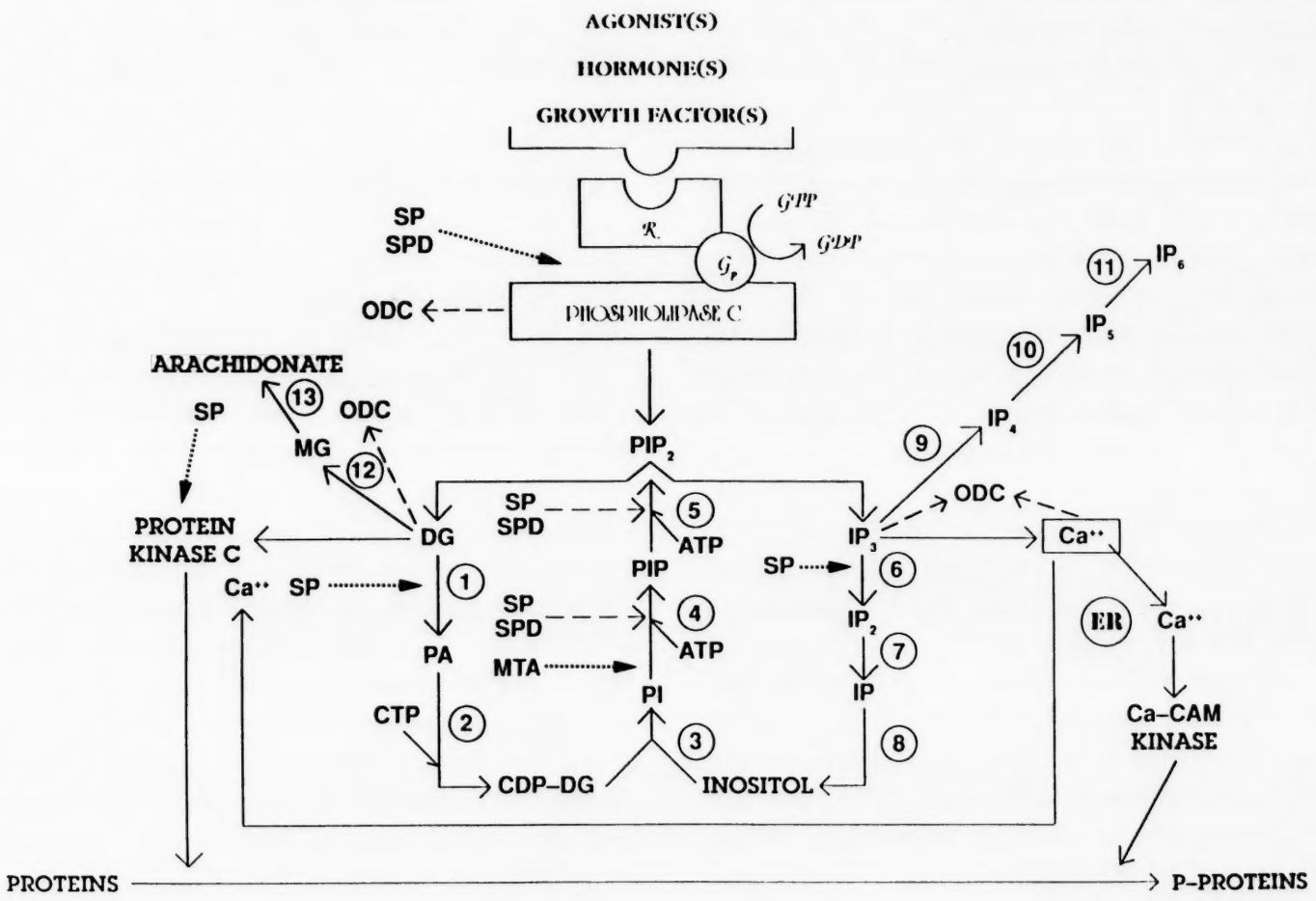

Fig. 1. Scheme showing the main steps of the polyphosphoinositide pathway (including recycling) (indicated by solid arrows). In the scheme the major nodes positively regulated by spermidine and/or spermine or their co-produced molecule (MTA) are indicated by dashed arrows; the major nodes negatively regulated by the same molecules are indicated by dotted arrows. In a similar way, some products of the polyphosphoinositide pathway (including recycling) are indicated as physiological inducers (dashed arrows) of ornithine decarboxylase activity. R = receptor; G_p = guanine nucleotide-regulatory binding protein; PI = phosphatidylinositol; PIP = phosphatidylinositol 4-phosphate; PIP_2 = phosphatidylinositol 4,5-bisphosphate; DG = 1,2-diacylglycerol; PA = phosphatidic acid; MG = monoacylglycerol; IP = inositol 4-monophosphate; IP_2 = inositol 1,4-bisphosphate; IP_3 = inositol 1,4,5-trisphosphate; IP_4 = inositol 1,3,4,5-tetraphosphate; IP_5 = inositol 1,3,4,5,6-pentaphosphate; IP_6 = inositol-esaphosphate; ER = endoplasmic reticulum; CAM = calmodulin; P-PROTEINS = phosphorilated proteins; ODC = L-ornithine decarboxylase (EC 4.1.1.17); SPD = spermidine; SP = spermine; MTA = 5′-deoxy-5′-methylthioadenosine; (1) DG kinase; (2) CDP-DG synthase; (3) CDP-DG inositol transferase (EC 2.7.8.11); (4) PI kinase (EC 2.7.1.67); (5) PIP kinase; (6) IP_3 phosphatase; (7) IP_2 phosphatase (EC 3.1.3.36); (8) IP phosphatase; (9) IP_3 kinase; (10) IP_4 kinase; (11) IP_5 kinase; (12) DG lipase (EC 3.1.1.34); (13) MG hydrolase.

growth is now compelling. It is therefore not surprising that polyamines have been demonstrated during recent years to also be involved in modulation of the phosphatidylinositol metabolism, although their exact roles in this regulation are far from clear. In this regard, it seems relevant to list the steps in phosphoinositide metabolism which are stimulated by or inhibited by polyamines, especially by spermidine and/or spermine, and to cite those products of phosphoinositide metabolism which have been shown to modulate ODC activity.

(1) Spermidine and spermine inhibit the G_p-protein-mediated activation of phosphoinositide hydrolysis catalysed by phospholipase C in GH_3 rat anterior pituitary tumor cells (Wojcikiewicz and Fain, 1988) and in human neutrophils (Das et al., 1987).

(2) Spermidine and spermine stimulate the activity of phosphatidylinositol-phosphodiesterase (phospholipase C) at low concentrations, but inhibit this reaction at higher concentrations (Eichberg et al., 1981; Sagawa et al., 1983).

(3) Exogenous phospholipase C induces ODC activity in guinea-pig lymphocytes in vitro and in mouse mammary gland explants (Kuramoto et al., 1983; Rillema et al., 1983; Otani et al., 1984; Jetten et al., 1985).

(4) Spermidine and spermine inhibit the phospholipid-sensitive Ca^{2+}-dependent protein kinase C (Wise et al., 1982; Qi et al., 1983; Levasseur et al., 1985; Moruzzi et al., 1987).

(5) Phosphatidylinositol-4-phosphate kinases are activated by spermidine and spermine in rat brain (Cochet and Chambaz, 1986; Lundberg et al., 1986), in rat liver (Lundberg et al., 1987) and in human polymorphonuclear leucocytes (Smith and Snyderman, 1988).

(6) IP_3 induces ODC activity in human T lymphocytes (Mustelin et al., 1986).

(7) DG induces ODC activity in mouse mammary gland explants (Rillema and Whale, 1988), in rat tracheal epithelial cells (Jetten et al., 1985) and in mouse epidermis (Smart et al., 1986, 1988; Hirabayashi et al., 1988), although some negative reports have been published (Otani et al., 1985; Kido et al., 1986).

(8) Spermine inhibits DG-kinase (Smith and Snyderman, 1988) and IP_3-phosphatase (Seyfred et al., 1984) and binds specifically to inositol phospholipids (Chung et al., 1985; Tadolini et al., 1985; Meers et al., 1986; Tadolini and Varani, 1986; Young and Green, 1986).

(9) Spermidine and spermine stimulate the phosphorylation of phosphatidylinositol in rat mast cells granules (Kurosawa et al., 1990).

In summary, separate investigations have been made of polyamine metabolism and phosphoinositide metabolism, which suggest that these two systems closely interact within mammalian cells. The concentrations of spermidine and/or spermine required to elicit these effects are well within the physiological range of these polycations. A scheme of phosphoinositide metabolism, including the steps regulated by spermidine and spermine and the metabolic steps influencing ODC activity is shown in Fig. 1.

3. Reflections and concluding remarks

The main aim of this review was not to define the role(s) of polyamines in the biochemical changes that occur in cells following hormonal exposure. Such a definition is impossible to provide at the present time, although it remains a highly desirable target for cellular and molecular endocrinology. The possibility that polyamines, especially spermidine and spermine, may be a part of the mechanism by which mammalian cells regulate their responses to extracellular signals and/or messengers is a very appealing one, and highly probable albeit by no means certain.

The conclusions clearly emerging from the data cited in this review are as follows. First, fine regulation of the polyamine biosynthetic pathway in cells involves both intracellular metabolic and extracellular hormonal factors; these two types of regulatory mechanisms appear to be closely interconnected.

Second, there is extensive evidence for the links between polyamines and hormones in mammalian cells and the functional importance of such links. It remains to be established what the nature and the limits of these links are. In this regard, it may be more convenient to begin by discussing the links between modulation of polyamine biosynthesis and the various extracellular signals which affect growth and/or differentiation of mam-

malian cells. A good example of this is the invariable coupling of polyamine biosynthesis to the interaction of a hormone or growth factor with its specific cell receptor, reflecting the close involvement of polyamines in the processes of cell growth and differentiation. In this respect, the polyamine biosynthetic pathway and the polyamines themselves can be properly considered as markers of hormone action. From this point of view, polyamines may be considered as necessary tools for, and therefore as crucial 'helpers' of hormones to express their effects on cell growth and differentiation. Therefore, we are in slight disagreement with Bolander (1989), who considers polyamines to be true 'second messengers' sensu stricto in the transduction of the signals of mammalian hormones, without taking into consideration the differences in the nature of the hormonal effects.

A key argument in favor of our hypothesis is that the effect (inhibitory or stimulatory) of those hormones on the polyamine biosynthetic pathway always parallels the effect (inhibitory or stimulatory) of these hormones on cell growth and differentiation. A second argument for our interpretation is that the concept of second messenger entails that the metabolic pathway producing this second messenger is invariably activated and stimulated by the interaction of the hormone with its receptor. This is not the case for the polyamine biosynthetic pathway, which can be stimulated or inhibited by a suitable hormone in its appropriate target organ and/or tissue, as mentioned above. Furthermore, by definition, a molecule that is a second messenger for a hormone should be able to mimic the effect of the hormone itself. Polyamines do not this, although they can be taken up by tissues (Seiler and Dezuere, 1990). The only exception are: (a) the insulin-like action of polyamines on adipose tissue, reported many years ago (Lockwood and East, 1974; Giudicelli et al., 1976), an antilipolytic action which has recently been further documented (Richelsen et al., 1989); (b) polyamines, like ACTH, strongly stimulate triacylglycerol lipase activity from rat brain (Le Petit et al., 1986); (c) polyamines, like antidiuretic hormone, stimulate Na^+/K^+-ATPase activity in the rat renal medullary thick ascending limb of Henle's loop (Charlton and Baylis, 1990). Therefore, it appears doubtful that polyamines can truly mimic hormonal action(s) as cyclic nucleotide derivatives do.

The traditional definition of second messenger molecule for a hormone includes the possibility of modulating the hormonal effects by appropriate drugs, which in turn can modulate the endogenous level of the second messenger. For the polyamine biosynthetic pathway there are several inhibitors available, some of which are considered specific for various regulatory points of the same pathway (reviewed in Scalabrino and Ferioli, 1982). The most specific and the most widely used polyamine inhibitors are α-difluoromethylornithine (DFMO), which inhibits ODC activity, and methylglyoxal bis-(guanylhydrazone) (MGBG), which is an inhibitor of SAMDC activity. We have summarized and listed in Table 2 the most significant results, both positive and negative, obtained with these. Table 2 is deliberately not as comprehensive as possible, inasmuch as we have omitted single reports on single hormone effect(s), which need to be better documented. Although the results in Table 2 appear almost random, two fundamental ideas emerge.

(a) The effect(s) of hormones and/or growth factors which are prevented by either of the aforementioned polyamine inhibitors include some of the biochemical cellular events induced by the hormones, growth factors and interleukins needed for cell growth and differentiation (Oka et al., 1978; Sakai et al., 1978; Goldstone et al., 1982; Seidel et al., 1985; Vincenzi et al., 1985; Willey et al., 1985; Bowlin et al., 1986; Marshall and Senior, 1986; Thyberg and Fredholm, 1987); on the other hand, some important completely opposite (i.e., non-inhibitory) results have been reported (Danzin et al., 1979; Henningsson et al., 1979; Bartolome et al., 1980; Pegg, 1981; Veldhuis et al., 1981; Rorke and Katzenellenbogen, 1984; Berger and Porter, 1986). Taken together, polyamine inhibitors have thus not been of great help in clarifying the role(s) of polyamines in the mechanism of action of mammalian hormones and/or growth factors.

(b) A peculiar example is the mammary gland, in which spermidine and spermine have been shown to be absolutely required for the appearance of the different effects of different hormones (prolactin, progesterone and insulin) on

TABLE 2

EFFECTS OF DIFFERENT POLYAMINE BIOSYNTHESIS INHIBITORS ON SOME CELLULAR BIOCHEMICAL EVENTS INDUCED BY HORMONES OR GROWTH FACTORS

Hormone or growth factor	Effect(s) prevented (polyamine inhibitor)	Ref. No.	Effect(s) NOT prevented (polyamine inhibitor)	Ref. No.
TS	↑ Renal weight (DFMO)	[65]	↑ Renal weight (DFMO)	[8]
	↑ Hexose and amino acid transport (DFMO)	[105]	↑ Prostate DNA and RNA content (DFMO)	[32]
	↑ Submaxillary gland protease activity (MGBG)	[86]	↑ Renal weight (MGBG)	[77]
ES	↑ Uterine weight (DFMO)	[143]	↑ Uterine weight (DFMO)	[193]
LH			↑ Granulosa cell progesterone production (DFMO)	[242,247]
			↑ Interstitial cell testosterone production (DFMO)	[168]
T_3 or T_4			Cardiac hypertrophy (DFMO)	[4,174]
PG	↑ Stomach DNA and RNA synthesis (DFMO)	[94,210]	↑ Colon DNA and RNA synthesis (DFMO)	[210]
PRL	↑ Mammary casein, lipid, lactose synthesis (MGBG)	[186,162b]		
Progesterone	↑ Endometrial glycogen synthesis (MGBG)	[52]		
Insulin	↑ Mammary DNA and RNA synthesis (MGBG)	[162,202]		
AVP	↑ ATPase activities renal medulla (DFMO)	[21b]		
CCK	↑ DNA polymerase ↑ RNA polymerase pancreas (DFMO)	[71b, 125c]		
EGF	↑ Growth rate of bronchial epithelial cells (DFMO)	[254]		
PDGF	↑ DNA synthesis in arterial muscle cells (DFMO) or (MGBG)	[235]		
	↑ DNA synthesis in 3T3 cells (DFMO)	[249]		
IL-2	↑ DNA synthesis in CTLL-20 cells (DFMO)	[17]		
IL-3	↑ DNA synthesis in FDC-P1 cells (DFMO)	[17]		

DFMO = α-difluoromethylornithine; MGBG = methylglyoxal bis-(guanylhydrazone); ↑ = increase.

this gland (Feil et al., 1977; Oka et al., 1978; Sakai et al., 1978; Rillema and Cameron, 1983), with similar results in a neoplastic mammary cell line (Linebaugh and Rillema, 1984). MGBG is therefore a successful inhibitor of the hormonal effects on normal mammary gland (Feil et al., 1977; Oka et al., 1978; Sakai et al., 1978; Rillema and Cameron, 1983; Oppat and Rillema, 1989).

Unfortunately, there is still no answer to questions of modulation of polyamine biosynthesis in target tissues by hormones that do not induce cell growth and/or differentiation in those tissues. Likewise, the biological significance of the activation of polyamine biosynthetic pathway by some hormones in tissues which are not considered traditional target(s) for these hormones is still to be established.

Finally, studies of polyamines and mammalian

hormones have clearly posed more problems than they have solved. For instance, the role(s) played by polyamines in the 'physiologically occurring' autocrine loops (Strober and James, 1988) as well as in the 'embryonically- or neoplastically-occurring' autocrine loops (reviewed in Scalabrino and Ferioli, 1989) are still quite obscure.

4. Is the mode of action of polyamines intracrine?

Evidence has been obtained very recently that some growth factors have an intracrine mode of action, in that they are intracellularly localized and act directly as intracellular messengers to modulate cellular functions, and thus that they do not need to be secreted to have their biological effects (Logan, 1990). From all that has been reported in the first part of this review, and obviously in the other reviews we have cited, it is quite clear that polyamines shows most, although not all, of the features that define intracrine action. Polyamines mainly act intracellularly and their biochemical and/or biological effects are achieved without being secreted. In fact, those polyamines that are secreted by the cells and thereafter circulate are completely devoid of any biological effect. Polyamines, unlike growth factors, do not immediately direct nuclear gene expression required to produce the biochemical and/or biological effects of growth factors. However, polyamines, because of their ability to interact with nucleic acids and to affect the synthesis and the functions of nucleic acids, act to modulate gene expression and in this way are required for appropriate gene expression induced by growth factors.

At present it is tempting to speculate that polyamines have an intracrine-like mode of action. If this hypothesis be substantiated in the future, it will greatly help to clarify the role of polyamines in the mechanism(s) of action of mammalian hormones and/or growth factors in eukaryotic cells.

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Inhibition of protein kinase C activity in cultured pituitary cells attenuates both cyclic AMP-independent and -dependent secretion of ACTH

Bernard Koch and Bernadette Lutz-Bucher

Institut de Physiologie, URA CNRS 309, 67084 Strasbourg Cedex, France

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Key words: Protein kinase C; Phorbol ester; Staurosporine; Corticotropin-releasing factor; Vasopressin; Veratridine

Summary

The present study examines the effect of reduction of protein kinase C (PKC) activity, as induced by either phorbol ester (PMA) down-regulation or staurosporine inhibition, on the secretion of ACTH from cultured anterior pituitary (AP) cells. Short-term (3 h) exposure of cells to 5 nM PMA resulted in almost complete desensitization to both PMA and vasopressin (AVP), while there was only a minor incidence on the effect of corticotropin-releasing factor (CRF). In contrast, long-term (12–24 h) exposure of cells to PMA, as well as pretreatment with staurosporine, dramatically reduced the stimulatory influence of CRF. This was shown not to be due to a decline in ACTH cells' stores, nor to the toxicity of phorbol ester or to a negative autofeedback of ACTH. Pretreatment of corticotrophs with PMA failed to dampen the CRF-induced cyclic AMP formation, while it caused a decline in the effects of forskolin and 8-bromoadenosine cyclic AMP. Stimulated ACTH secretion subsequent to either veratridine- or high K^+-induced cell depolarization was likewise decreased. We conclude that in corticotrophs the stimulatory action of not only AVP, but also of that of CRF on ACTH secretion strongly relies on PKC activity. In the case of CRF, however, this may not be a primary consequence of receptor occupation, as evidence suggests an indirect relationship which may involve PKC regulation of Ca^{2+} channels and/or the ion's intracellular messenger function.

Introduction

Protein kinases C (PKCs) appear to be involved in the transduction system of a variety of cellular activities, including cell growth and differentiation, as well as oncogene and hormone actions (Takai et al., 1984). The enzyme is activated by diacylglycerol, which is generated by receptor-induced phosphatidylinositol phosphate hydrolysis, together with inositol trisphosphate; a messenger promoting calcium mobilization from intracellular stores (Berridge, 1987). PKC may also be activated via other cellular routes and by tumor promoting phorbol esters (Huang, 1989). However, while short-term exposure of cells to the latter drugs actually enhances PKC activity, long-term exposure can lead to its down-regulation and desensitization in a number of cell types (Phillips and Jaken, 1983; Bileszikjian et al., 1987; Thams et al., 1990).

Regulation by secretagogues of peptide secre-

Address for correspondence: Bernard Koch, Institut de Physiologie, URA CNRS 309, 21, rue René Descartes, 67084 Strasbourg Cedex, France.

tion from pituitary corticotrophs relies on processes that are both dependent on and independent of cyclic AMP production (Antoni, 1986). Corticotropin-releasing factor (CRF) and β-adrenergic agonists stimulate ACTH secretion primarily through activation of adenylate cyclase, while the effects of vasopressin (VP) and angiotensin appear to involve the inositol-phospholipid-PKC pathway (Raymond et al., 1985; Guillon et al., 1987). Similarly, phorbol esters have been shown not only to stimulate peptide output from pituitary cells, but also to enhance both CRF-induced accumulation of cyclic AMP and ACTH secretion (Heisler, 1984; Cronin et al., 1986; Abou-Samra et al., 1987; Lutz-Bucher et al., 1990).

In apparent contradiction with some of these notions, we have previously reported that PKC inhibition, due to enzyme depletion and retinal inhibition, not only blunted the stimulatory effect of arginine vasopressin (AVP), but unexpectedly, also that of CRF (Koch and Lutz-Bucher, 1989). This suggested that the mechanism of action of CRF, which, unlike that of AVP, primarily involves cyclic AMP as a second messenger, also strongly relies on the PKC pathway.

In the present study we have extended and strengthened these findings further by examining the kinetics of the effect of PMA exposure of rat anterior pituitary (AP) cells on AVP- and CRF-stimulated ACTH secretion, as well as on CRF-induced cyclic AMP formation. Moreover, we aimed at ascertaining whether PMA-induced inhibition of hormone output may possibly be accounted for by some artifactual or indirect effects and, also, if PKC desensitization may affect hormone release triggered by various other secretagogues. Finally, in addition to using PKC-depleted cells, we also meant to examine the influence of inhibition of PKC activity by means of the potent inhibitor staurosporine, which is thought to interact with the catalytic subunit of the enzyme (Tamaoki et al., 1986).

Materials and methods

Pituitary tissues and reagents

Pituitary glands were dissected out from Wistar male rats (300–350 g) and separated between AP and neurointermediate lobes. Antisera against ACTH and cyclic AMP were generous gifts of Ch. Oliver (Marseilles, France) and G. Pelletier (Québec, Canada), respectively. Phorbol 12-myristate 13-acetate (PMA), 4α-phorbol 12,13-didecanoate (PDD), staurosporine, veratridine, forskolin, 8-bromoadenosine 3′,5′-cyclic monophosphate and Dulbecco's modified Eagle's medium (DMEM; phenol red-free) were purchased from Sigma (St. Louis, MO, U.S.A.). CRF and AVP were from Peninsula (Merseyside, U.K.). Phorbol esters, staurosporine (dissolved in dimethyl sulfoxide), veratridine and forskolin (dissolved in ethanol) were added to incubation media as a 2000-fold concentrated stock solution. The same amount of vehicle (final concentration of 0.05%) was added to control dishes.

Pituitary cell cultures

AP tissues were enzymatically dispersed and dissociated cells were purified as already reported (Koch and Lutz-Bucher, 1989). Cells were plated at a density of $2–3 \times 10^5$ cells/well in 24-well plates and cultured for 3 days in DMEM, supplemented with 7.5% horse serum and 2.5% fetal calf serum and antibiotics. One day prior to the experiments standard medium was changed for DMEM containing charcoal-dextran-treated sera (10%) and either PMA or vehicle for the time periods indicated. The possibility that phorbol ester (used at a low concentration of 5 nM) could have deleterious effects on the cells was tested and precluded by the observations that (i) the inactive compound phorbol ester PDD left the secretory capacity of the corticotrophs unaffected compared to control cells (Table 2); (ii) cell exposure to PMA did not alter CRF-induced cyclic AMP production (Fig. 4); (iii) treated cells retained their ability to exclude trypan blue to the same extent as did control cells.

Cell incubation and peptide secretion

Cells were washed in Krebs-Hepes (KH) buffer composed of (in mM): 127 NaCl, 4.7 KCl, 2.5 $CaCl_2$, 1.2 KH_2PO_4, 1.2 $MgSO_4$, 20 Hepes, 0.1 ascorbic acid and enriched with 0.2% glucose, 0.02% glutamine, 0.1% bovine serum albumin (w/v) and 0.5% (v/v) amino acid mixture (50 ×). They were then equilibrated for 1 h in KH buffer and further incubated, at 37 °C and under O_2, in

the presence or absence of various secretagogues as indicated. At the completion of the incubation period (1–3 h), media were centrifuged (1000 × *g*/10 min) to sediment any floating cells and aliquots of the supernatants were kept frozen at −25 °C until assayed for ACTH content, as previously described (Koch and Lutz-Bucher, 1989).

Cell production of cyclic AMP

After being washed and preincubated in KH buffer as indicated above, cells were incubated for 15 min in the presence or absence of various concentrations of CRF and 0.5 mM 3-isobutyl-1-methylxanthine (IBMX). The reaction was stopped by changing the incubation medium to ice-cold 0.1 M HCl. The incubation media were centrifuged (1000 × *g*/10 min) and the supernatants acidified with 10% (v/v) 1 M HCl, while the cells were disrupted by a freezing-thawing cycle, followed by sonication. The cell extracts were then centrifuged at 10,000 × *g* for 10 min and the resulting supernatants saved at −25 °C for cyclic AMP measurement by radioimmunoassay (RIA) (Koch and Lutz-Bucher, 1989).

Statistics

Statistical analysis was performed by analysis of variance, followed by Duncan's multiple range test. A rejection level of $P < 0.05$ was considered significant. The half-life ($T_{1/2}$) disappearance of the cell's responsiveness to secretagogues was calculated as the ratio: −ln 2/slope of the regression line, on a semi-log scale. Data displayed in the figures are means ± SE of 3–6 observations and are representative of 2–3 identical experiments, which yielded qualitatively identical results.

Results

Time-course of the effect of PMA pretreatment

The kinetics of the ACTH response to PMA, after pre-exposure of the AP cells to the phorbol ester (5 nM) for various time periods, showed a biphasic pattern: a rapid desensitization during the first 3 h of pretreatment, followed by a much lower rate of decrease of the cell's responsiveness (Fig. 1). The corresponding $T_{1/2}$ were calculated to be 2.4 h and 13 h, respectively.

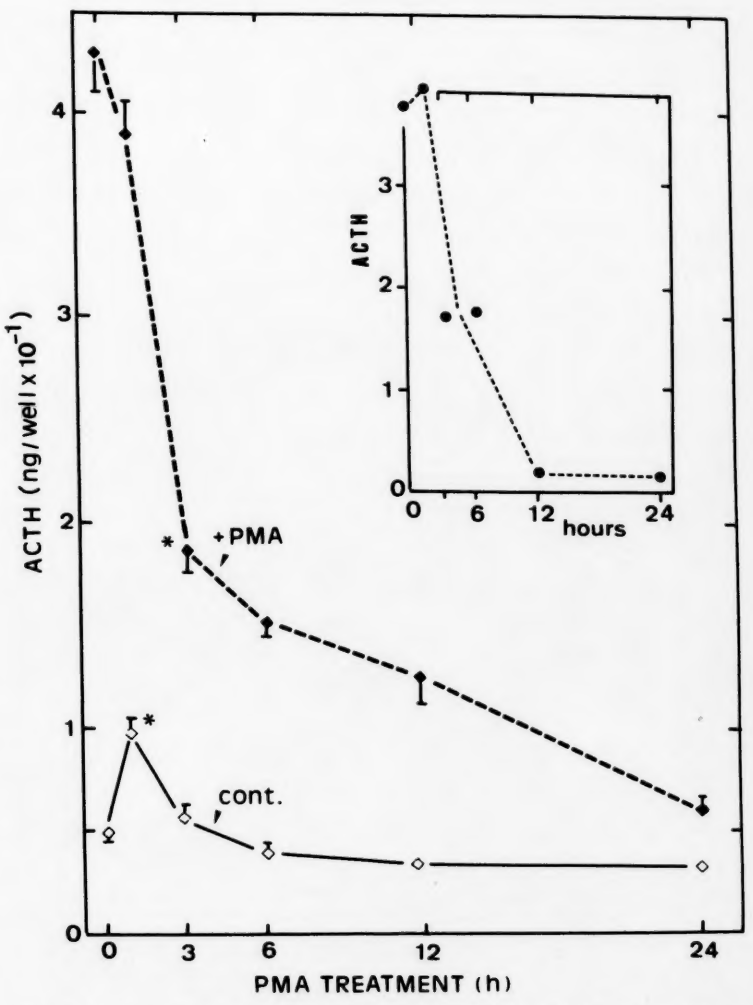

Fig. 1. Time course of the effect of PMA (5 nM) pretreatment of cells on subsequent PMA stimulation (1 nM) of ACTH secretion during a 3 h incubation period. Each value is the mean ± SE of three observations done in duplicate. * Different from non-treated cells ($p < 0.05$). Inset: points represent net increases of ACTH secretion (stimulated minus basal levels) corrected for tissue concentration of ACTH. Cells incubated with PMA (◆) or vehicle (◇).

Exposure of AP cells to 5 nM PMA for 3 h caused a nearly complete dampening of AVP-induced ACTH secretion (Fig. 2, bottom panel), with a $T_{1/2}$ value of 2.2 h, closely similar to the value of the initial component of the disappearance rate of the PMA response. In sharp contrast with the latter findings, we found that the rate at which the cells were rendered unresponsive to CRF was about 3 times lower compared to AVP, with a calculated $T_{1/2} = 7$ h for the time period 0–12 h (Fig. 2, top panel). After 12–24 h of PMA exposure, however, the stimulatory action of CRF was blunted by 70–80%, whether the cells were challenged with CRF for 3 h (Fig. 2) or for 1 or 2 h (not shown).

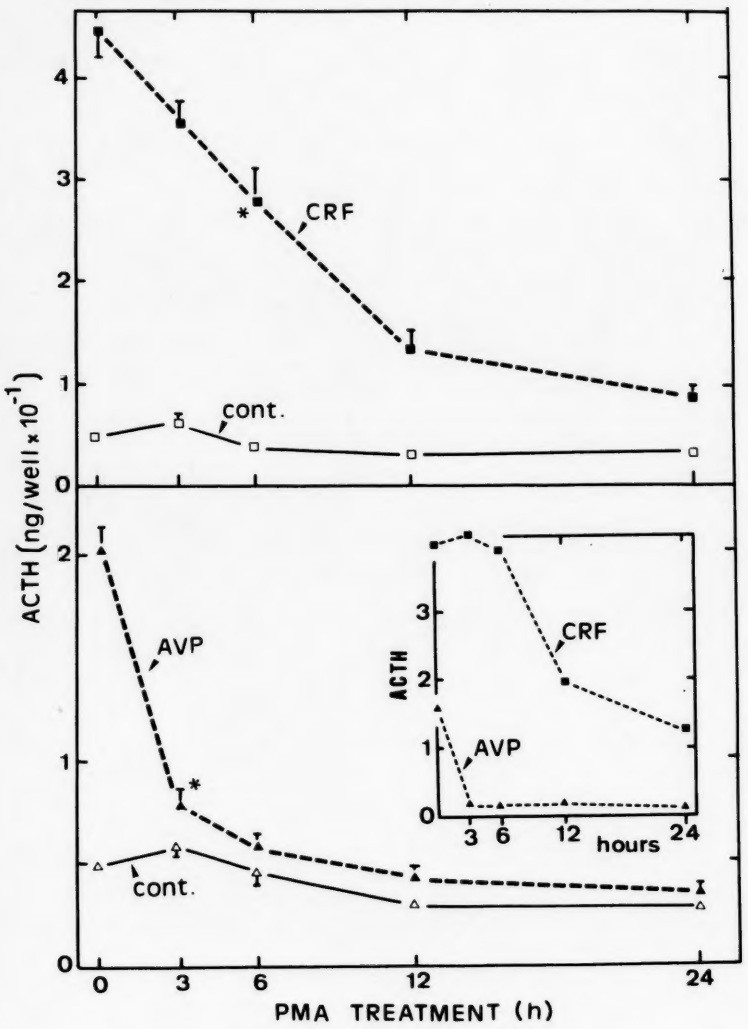

Fig. 2. Time course of the effect of PMA pretreatment (5 nM) on ACTH secretion induced by CRF (1 nM; upper panel) and AVP (10 nM; lower panel). The incubation time with peptides was 3 h. Each value is the mean ± SE of three observations done in duplicate. * Different from non-treated cells ($p < 0.05$). Inset: points represent net increases of peptide secretion (stimulated minus basal levels) corrected for tissue concentrations of ACTH. Cells incubated with CRF (■) and AVP (▲) or vehicle (corresponding open symbols).

Influence of cellular ACTH content

Because long-term exposure to PMA, a potent secretagogue of ACTH, lowers cellular hormonal content (up to about 50% at 24 h) and may thus cause a reduction in the cell's response independently of PMA-induced PKC depletion, we expressed the former data as a function of the amount of ACTH available. As shown in the insets to Figs. 1 and 2, the net increase (total minus basal secretion) of ACTH output (corrected for cellular peptide contents) due to PMA and AVP was nearly abolished at, respectively, 12 h and 3 h of PMA pretreatment. The stimulatory effect of CRF was attenuated by 51% and 68% at 12 h and 24 h, respectively, though there was no detectable influence for exposure times of up to 6 h. Thus, our observations as a whole are valid not only on the basis of absolute, but also of relative secretions of ACTH.

Autofeedback of ACTH and effect of PDD

We next determined if the inhibitory influence of PMA exposure on hormonal output could be due to either a negative feedback control of ACTH on its proper release (because PMA exerts a long-lasting stimulatory action on peptide secretion) or eventually to a cytotoxic effect of the phorbol ester itself. To test these possibilities, AP cells were exposed for 18 h to increasing concentrations of ACTH or to the inactive phorbol ester PDD before being challenged with various secretagogues. As shown in Tables 1 and 2, none of these treatments significantly affected either basal or stimulated secretion of ACTH elicited by PMA, CRF and AVP.

Staurosporine inhibition of PKC activity

Results obtained with cells depleted of PKC were further confirmed by inhibiting enzyme activity with staurosporine. Exposure of AP cells to the inhibitor caused a dose-dependent attenuation of both PMA- and CRF-induced ACTH secretion (Fig. 3), with half-maximal inhibitions being achieved with about 30 and 90 nM staurosporine,

TABLE 1

EFFECT OF ACTH PRETREATMENT ON THE SECRETORY ACTIVITY OF AP CELLS

Cells were exposed during 18 h to various concentrations of ACTH. They were then washed twice and preincubated in ACTH-free buffer as described in Materials and Methods and further tested for their ability to secrete ACTH in response to 10 nM AVP and 1 nM of either CRF or PMA (3 h incubation period). Data (ACTH concentrations in ng/well) are means ± SE of three wells and are representative of two identical experiments.

Pretreatment	Control	AVP	CRF	PMA
None	2.1 ± 0.1	9.0 ± 0.3	25.3 ± 0.6	20.9 ± 0.6
ACTH				
1 nM	2.4 ± 0.1	7.1 ± 0.5	25.6 ± 1.1	20.7 ± 1.1
10 nM	2.5 ± 0.3	8.3 ± 0.8	27.7 ± 1.3	21.2 ± 1.4
100 nM	3.1 ± 0.1	8.5 ± 0.5	26.1 ± 1.8	21.0 ± 1.0

TABLE 2

EFFECT OF LONG-TERM EXPOSURE OF AP CELLS TO THE INACTIVE PHORBOL ESTER PDD

Cells were incubated with 5 nM PDD for 18 h and their ACTH response to 1 nM of either PMA or CRF tested during a 3 h incubation period. Each value (ACTH concentrations in ng/well) is the mean ± SE of four wells and data are representative of two such experiments.

Pretreatment	−PDD	+PDD
Vehicle	3.7 ± 0.2	3.2 ± 0.4
PMA	12.6 ± 0.8	14.8 ± 1.2
CRF	22.6 ± 0.9	19.1 ± 0.7

respectively. The drug, at a dose of 1 μM, was found to consistently abolish the stimulatory effect of PMA on ACTH secretion, while it dampened by only about 70% that of CRF, whether the cells were exposed to the secretagogues for time periods of 1, 2 or 3 h (not shown). Finally, it should be noted that the inhibitor had no effect on the cell's stores of ACTH: 57.2 ± 4.1 vs. 60.0 ± 2.8 ng/well in the absence and presence of 1 μM staurosporine, respectively ($n = 6$). Nor did the drug change the cyclic AMP response to various concentrations of CRF (not shown), indicating that the pretreatment did not damage the cells.

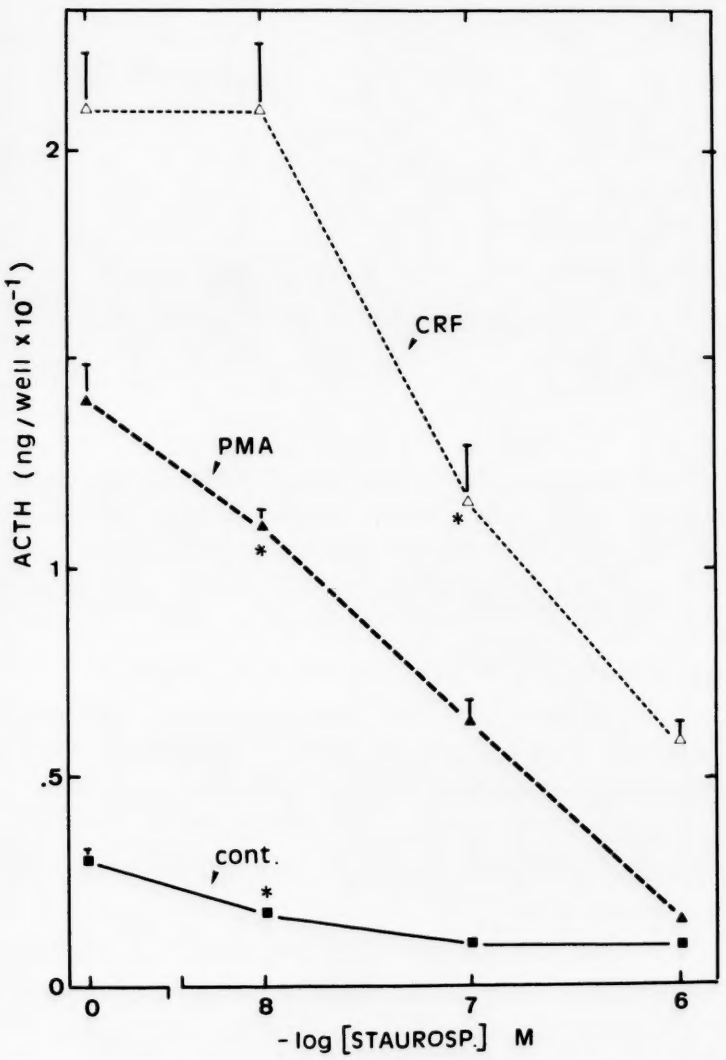

Fig. 3. Dose-dependent effect of staurosporine on ACTH secretion stimulated by 1 nM of either PMA or CRF. Cells were exposed for 30 min to the inhibitor and further incubated with peptides for 3 h. Similar results were obtained with incubated times of 1 and 2 h. Each point is the mean ± SE of three observations done in duplicate. * Different from non-treated cells ($p < 0.05$). Cells incubated in the presence of CRF (△) and PMA (▲). Control cells (cont.).

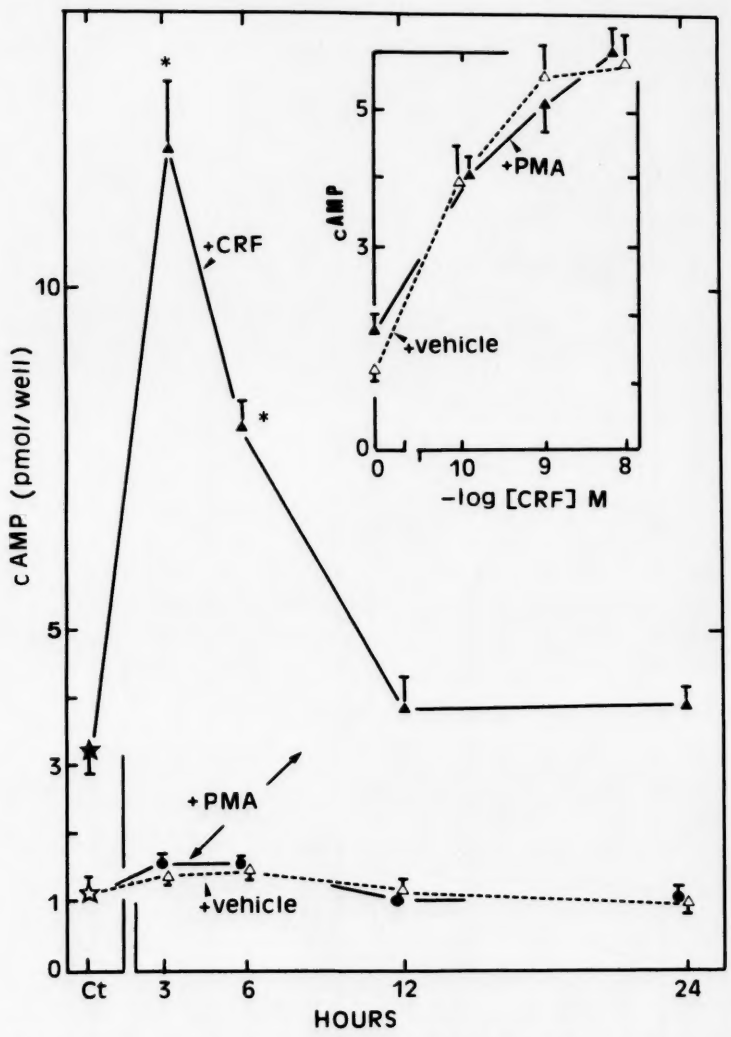

Fig. 4. Time course of the effect of PMA (5 nM) or vehicle on tissue cyclic AMP concentrations elicited by CRF (1 nM). Incubation was performed in the presence of CRF and 0.5 mM IBMX for 15 min. Inset: Effect of increasing concentrations of CRF on vehicle- and PMA-treated (5 nM/18 h) AP cells (open and dark symbols, respectively). Ct: control, vehicle-treated, cells. Each value corresponds to the mean ± SE of six observations. Cells incubated in the absence (△) and presence of CRF (▲).

Cell production of cyclic AMP

We next meant to ascertain if PMA pretreatment of cells did affect the formation of cyclic AMP, the second messenger primarily involved in the effect of CRF. Data in Fig. 4 illustrate the time-course of cyclic AMP production stimulated by 1 nM CRF in AP cells pretreated with PMA or vehicle for 3–24 h. It appears that cells previously exposed to PMA for 3–6 h showed a dramatic enhancement in their capacity to accumulate cyclic AMP in response to CRF (3- to 4-fold the value observed in control cells). However, after exposure times of 12–24 h cellular cyclic nucleotide production returned to control values. This is also apparent in the inset to Fig. 4, which shows that there was no significant difference of second messenger formation in cells stimulated by increasing

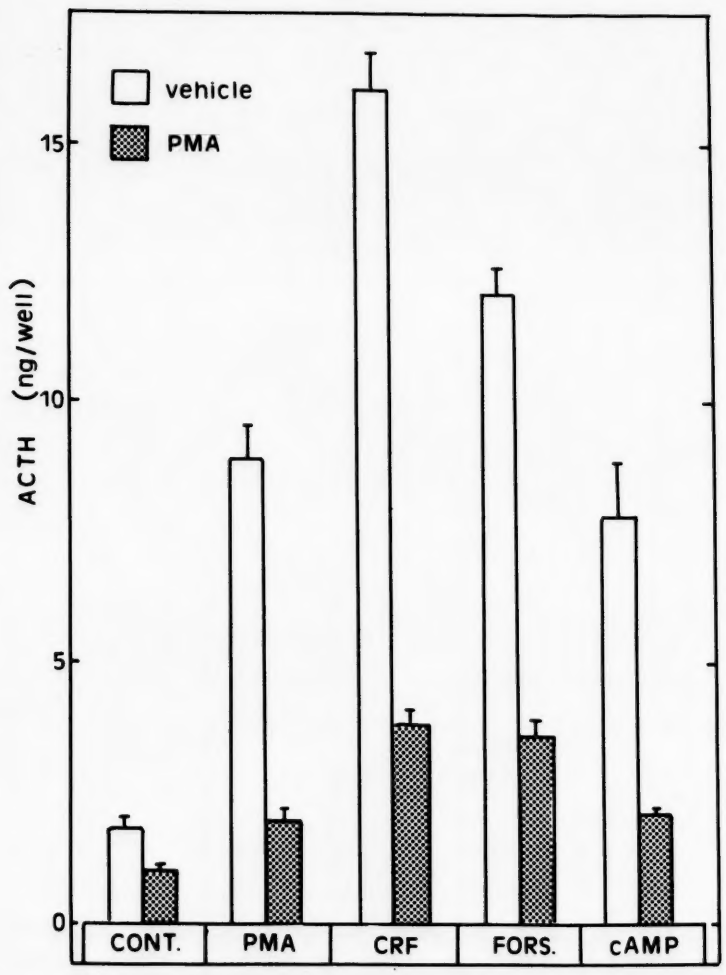

Fig. 5. Effect of cell exposure to PMA (5 nM/18 h) or vehicle on the ACTH responses to PMA (1 nM), CRF (1 nM), forskolin (FORS.; 10 μM) and 8-bromoadenosine cyclic AMP (cAMP; 1 mM) during a 3 h incubation period. Each bar corresponds to the mean ± SE of six observations. Values referring to PMA-treated cells are all significantly different from respective control cells ($p < 0.05$).

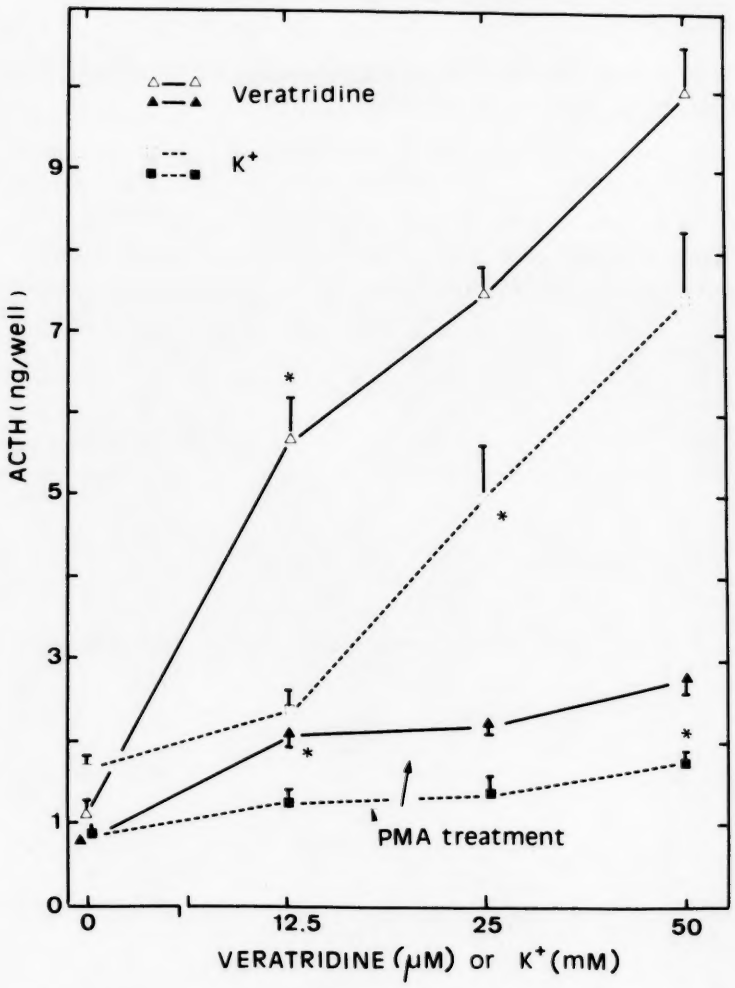

Fig. 6. Influence of exposure of AP cells to PMA (5 nM/18 h) or vehicle on the secretory responses to increasing concentrations of veratridine or KCl. Each value is the mean ± SE of three observations done in duplicate. * Different from control cells ($p < 0.05$). Open and dark symbols stand for vehicle- and PMA-treated cells, respectively.

concentrations of CRF, whether treated with PMA or vehicle for 18 h. Nor were there any significant differences in the cyclic AMP contents of corresponding incubation media (not shown).

Response of PKC-depleted cells to forskolin and 8-bromo-cyclic AMP

In order to bypass CRF–receptor interaction, intracellular messenger concentration was elevated by directly activating adenylate cyclase with forskolin and by incubating cells in the presence of 8-bromo-cyclic AMP. As depicted in Fig. 5, these agents triggered marked increases (4–6 times the basal value) of ACTH output from control AP cells (vehicle). Previous exposure of cells to PMA caused 74% and 80% reductions in the net stimulatory effects (total minus respective control values)

of forskolin and 8-bromo-cyclic AMP, respectively. These values were closely similar to that observed with PMA (86%) and CRF (80%).

Effect of veratridine and K^+ on ACTH secretion from kinase C-depleted cells

Given the facts that ACTH secretion from corticotrophs closely relies on the presence of extracellular Ca^{2+} (Antoni, 1986) and that PKC may be involved in phosphorylation and regulation of ion channels (Kaczmarek, 1987), we tested the effect of cell depletion of PKC on hormone secretion elicited by agents which actually cause opening of Ca^{2+} channels by depolarizing the cell. Both veratridine (12.5–50 μM) and KCl (25–50 mM) caused dose-related increments of ACTH secretion from control AP cells, which ranged from 5 to 9 times and 3 to 4.5 times the basal values, respectively (Fig. 6). Long-term exposure to PMA rendered the cells almost unresponsive to these agents, with ACTH secretions declining by 70–80%, depending on the concentrations of the secretagogues being used.

Discussion

The present study, in which two methods were used to dampen PKC activity in cultured AP cells (phorbol ester-induced down-regulation and staurosporine inhibition of enzyme activity), clearly points to a critical role for PKC in the secretory processes stimulated by both AVP and CRF in corticotrophs. However, PKC depletion exerted differential effects on stimuli such as AVP, which directly involves the inositol-phospholipid-PKC pathway and those like CRF, which activates adenylate cyclase (Raymond et al., 1985; Antoni, 1986). The present kinetic studies showed that, in the case of AVP, ACTH secretion decreased in parallel with the decrement of the cell's responsiveness to the PKC activator PMA, with initial rapid components showing half-lives of 2–2.5 h. Peptide secretion stimulated by AVP was almost completely abolished with only about a 50% drop in the corticotroph's PKC activity (as judged by the responsiveness to PMA), suggesting a direct key role for PKC in the mechanism of action of vasopressin. This is in accord with previous similar findings (Bileszikjian et al., 1987). The corticotroph's response to CRF, on the other hand, fell less rapidly ($T_{1/2}$ of the initial component was 7 h) and, in contrast to AVP, showed only about a 20% decrease at a time when PMA pretreatment already caused half-maximum inhibition of phorbol ester stimulation. After 12–24 h of cell exposure to PMA, however, there was a dramatic 70–80% inhibition of ACTH secretion elicited by CRF. This suggests that PKC also plays a major, albeit most probably indirect, function in the action of CRF on corticotrophs. It is of interest to note that the GnRH stimulation of gonadotropin secretion from cultured AP pituitary cells likewise appeared to decline in PMA-treated cells, as well as after staurosporine inhibition, although, surprisingly, the short-term effect (20 min) of GnRH was not reduced in PKC-depleted cells (Stojilkovic et al., 1989; Dan-Cohen and Naor, 1990). In this case, however, evidence indicates agonist-induced phosphoinositide breakdown (Conn et al., 1987; Morgan et al., 1987; Naor, 1990).

In order to examine the possibility of these data being due to some artifactual effects relative to long-term treatment of cells with PMA, we performed the following control experiments. First, taking into account the fact that under these conditions there was a drop in the cellular concentration of ACTH, we expressed data as a function of cell hormone content and found this to confirm our results based on absolute values. In addition, we showed in a previous study that PMA did not affect mRNA levels of proopiomelanocortin, a precursor for ACTH formation (Lutz-Bucher et al., 1989). Second, an eventual cytotoxic effect of the phorbol ester was considered. However, such an effect of PMA, which was used at a dose at least two orders of magnitude lower than in similar studies on the mechanism of action of GnRH (Stojilkovic et al., 1989; Dan-Cohen and Naor, 1990), was precluded by the observation that the inactive compound PDD failed to affect ACTH secretion. Third, a possible negative autofeedback of ACTH on its proper release was likewise excluded, for we demonstrated that long-term exposure of AP cells to high concentrations of ACTH did not hamper the cell's responsiveness to stimuli. Finally, using staurosporine (the most specific inhibitor of PKC available) to block enzyme activ-

ity, we confirmed our data based on PMA-induced PKC depletion.

The findings that blockade of PKC in cultured AP cells reduces long-term stimulatory effects of GnRH on LH release, but not acute ones, led Dan-Cohen and Naor (1990) to consider the existence of a biphasic secretory response to the neuropeptide. Similarly, the effect of AVP on ACTH secretion from perifused AP cells has been shown to present a first rapid phase, followed by a long-lasting period, while, in contrast, the action of CRF, though rapid in its onset, produced a plateau of peptide secretion (Won and Orth, 1990). It is clear that the static incubation conditions used in the present study, as in studies aimed at elucidating the mode of action of GnRH, obviously refers to a sustained phase of peptide release. We showed that although the AVP- and CRF-stimulated secretory activities were similarly reduced in long-term PMA-treated (PKC-depleted) corticotrophs, a clear-cut difference in the action of these stimuli was revealed after short-term exposure to the phorbol ester. Indeed, cells desensitized much more rapidly to the effects of both PMA and AVP than to that of CRF, reflecting differences in the transduction signals being involved. Thus if the secretory response to AVP seems to mainly rely on the Ca^{2+}-phosphoinositide-PKC pathway, the response to CRF appeared to be rather more complicated and, at least in the initial period of phorbol ester pretreatment, was in great part independent of PKC. Using a different experimental approach, a very recent study (Oki et al., 1990) reports that PKC-depleted AP cells, when perifused with peptides during short periods of time (10 min), showed the second phase of the ACTH response to AVP and the synergism of the latter peptide with the CRF effect to be reduced, while there was no change in the secretory response to CRF alone. These observations, somehow, support the present evidence that short-term (3 h) exposure of cells to PMA actually blocked the effect of AVP, but only slightly and nonsignificantly ($p > 0.05$) affected that of CRF.

It thus seems that the chain of cellular events triggered by long-term CRF stimulation involves, in some way, PKC activation. However, while in a different type of cells, namely Leydig cells, CRF actually did stimulate PKC activity (Ulisse et al., 1990), it is most likely that the effect of CRF on corticotrophs relies in an indirect manner to the enzyme. Similarly, in a recent review devoted to the epidermal growth factor (EGF), it has been suggested that PKC may be indirectly stimulated upon EGF–receptor interaction (Carpenter and Cohen, 1990). PKCs may act at multiple sites in the receptor-mediated signalling pathway and could do so by controlling phosphorylation of key cellular substrates, such as, for example, receptors and/or coupled G-proteins or components of ion channels (Takai et al., 1984; Huang, 1989). In the case of CRF-receptor activation, the enzymes may affect the very peptide–receptor interaction, as well as various post-receptor events, including the cell's production of arachidonic acid and its metabolites (via the generation of phosphatidylcholine, Huang, 1989; Pelech and Vance, 1989), which may act as ACTH releasing factors (Vlaskovska and Knepel, 1984; Abou-Samra et al., 1986). Also, PKC and phorbol esters are likely to regulate gene expression by both acting on the cyclic AMP response element (Goodman, 1990).

In an attempt to somewhat clarify these issues, we determined the kinetics of CRF-mediated cyclic AMP production in PMA-pretreated cells and analysed the effect of bypassing the receptor by stimulating these cells with forskolin (which activates adenylate cyclase) or 8-bromoadenosine-cyclic AMP. Furthermore, we examined the responsiveness of PKC-depleted corticotrophs to veratridine (a Na^+ channel activator) and high K^+ concentrations, that is, to agents that depolarize the cells and cause Ca^{2+} entry. First, we showed that long-term exposure of AP cells to PMA failed to significantly alter CRF-stimulated cyclic AMP formation, while acute exposure resulted in the previously described enhancement of the action of CRF (Cronin et al., 1986; Abou-Samra et al., 1987). In addition, we report here this effect to be a long-lasting one that continued for at least 6 h. An interesting fact is that at that time point, when the cyclic nucleotide concentration yet was 2.5 times the control level, the ACTH response to CRF was already significantly reduced, thereby strongly suggesting a blockade downstream to the second messenger formation. This view gains support from the observation that peptide secretion triggered by forskolin and 8-

bromoadenosine-cyclic AMP was blocked in PKC-depleted cells, as well. Moreover, we provide evidence that the stimulatory influence of veratrine and K^+ on ACTH output was likewise reduced, suggesting that the loss of PKC activity impaired Ca^{2+} influx through ion channels and hence the Ca^{2+} intracellular messenger system, as already documented in other cell types (Kaszmarek, 1987; Graff et al., 1989).

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Rapid stimulation of calcium uptake and protein phosphorylation in isolated cardiac muscle by 1,25-dihydroxyvitamin D_3

Juana Selles and Ricardo Boland

Departamento de Biologia, Universidad Nacional del Sur, 8000 Bahia Blanca, Argentina

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Key words: Calcium uptake; Protein phosphorylation; Cardiac muscle; 1,25-Dihydroxyvitamin D_3

Summary

1,25-Dihydroxyvitamin D_3 has been shown to induce rapid changes in calcium fluxes in skeletal muscle and other target tissues independently of gene activation. The possibility that the hormone would produce similar effects in heart where 1,25-dihydroxyvitamin D_3 receptors and activities have been shown, was studied. A significant increase of ^{45}Ca uptake by left ventricular slices from vitamin D-deficient chicks was observed upon incubation for 1–10 min with physiological doses of 1,25-dihydroxyvitamin D_3. This stimulation was dose-dependent and specific for the hormone when compared with vitamin D_3, 25-hydroxyvitamin D_3 and 24,25-dihydroxyvitamin D_3 and could not be associated to changes in lipid synthesis as assessed by measurements of [^{3}H]glycerol incorporation into cardiac tissue lipids. The Ca channel blockers nifedipine (30 μM) and verapamil (10 μM) abolished the increase in Ca uptake produced by 1,25-dihydroxyvitamin D_3. The rapid effects of the hormone on heart Ca influx were accompanied by a stimulation of the phosphorylation of two microsomal proteins of 43 kDa and 55 kDa. These results further support a direct action of 1,25-dihydroxyvitamin D_3 in the regulation of cardiac muscle Ca metabolism which may involve activation of Ca channels.

Introduction

1,25-Dihydroxyvitamin D_3 ($1,25(OH)_2D_3$) plays a major role in the regulation of the extracellular calcium homeostasis (Norman, 1979). It has been shown that $1,25(OH)_2D_3$ is also a potent inducer of cell differentiation (Abe et al., 1981; Tanaka et al., 1982) and participates in the regulation of intracellular calcium in skeletal muscle (Boland, 1986). It is well established that $1,25(OH)_2D_3$ acts as a classical steroid hormone via a receptor-mediated nuclear mechanism of action (Norman et al., 1982; Minghetti et al., 1988). In addition, it has been reported that the sterol exerts rapid actions in classical as well as non-classical target tissues independent of gene activation (Nemere et al., 1984; de Boland and Boland, 1987; Lieberherr, 1987; Nemere and Norman, 1987; Baran and Kelly, 1988). Evidence has been obtained indicating that these non-genomic effects of $1,25(OH)_2D_3$ may be mediated by the activation of cell membrane voltage-dependent Ca^{2+} channels (de Boland and Boland, 1987; Caffrey and Farach-Carson, 1989; de Boland et al., 1989; Tornquist and Tashjian, 1989) and/or through the

Address for correspondence: Ricardo Boland, Departamento de Biologia, Universidad Nacional del Sur, 8000 Bahia Blanca, Argentina.

phosphatidylinositol phosphate/protein kinase C pathway (Tang et al., 1987; Wali et al., 1970).

A relationship between vitamin D and heart function has been recently suggested by animal model studies (Weishaar and Simpson, 1987, 1989; Hochhauser et al., 1988). Cardiac muscle activity is highly dependent on intracellular Ca^{2+}. Walters et al. (1987) reported that direct treatment of cultured heart ventricular cells with $1,25(OH)_2D_3$ for 24 h results in an enhancement of cell Ca^{2+} uptake which is blocked by prior addition of RNA and protein synthesis inhibitors. In agreement with these observations, the presence of a receptor specific for $1,25(OH)_2D_3$ in cultured cardiac cells has been shown (Simpson et al., 1985; Walters et al., 1986).

The objective of the present work was to investigate whether $1,25(OH)_2D_3$ exerts in cardiac muscle, similarly as in other target tissues, rapid effects on Ca^{2+} fluxes and to obtain information about the possible mechanism involved.

Materials and methods

Chemicals

$1,25(OH)_2D_3$ was kindly donated by Dr. M. Uskokovic (Hoffman-La Roche Co., Nutley, NJ, U.S.A.). ^{45}Ca and [^{32}P]ATP were purchased from New England (Boston, MA, U.S.A.). Verapamil hydrochloride was obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Nifedipine and nitrendipine were a gift from Bayer Co. (Leverkusen, F.R.G.).

Animals

Chicks were fed with a vitamin D-deficient diet containing 1.6% calcium and 1.0% phosphorus (Wasserman and Taylor, 1973) from hatching and during 5 weeks. Animals were maintained in an environment deprived of ultraviolet light. Serum $25OHD_3$ levels, determined by a protein binding method (Norman and Roberts, 1980), were not detectable (less than 4 ng/ml). Serum Ca and P concentrations were 8.70 ± 0.50 and 9.18 ± 0.41 mg/100 ml, respectively.

Cardiac muscle preparations and in vitro treatment

The animals were sacrificed by decapitation. Their hearts were immediately removed and collected in ice-cold Krebs-Henseleit solution containing 0.5 mM Ca. The left ventricles were cut in slices of 1–1.5 mm thickness and 15 mm length and transferred to a Krebs-Henseleit solution containing 2% glucose and 0.5 mM $CaCl_2$. The tissue was preincubated for 20 min at 20 °C under 95% O_2-5% CO_2. $1,25(OH)_2D_3$ was added as ethanolic solution at the concentrations indicated for each experiment. An equal amount of ethanol was added to control samples. When calcium channel blockers were tested, they were added to the medium dissolved in ethanol 5 min prior to sterol treatment. In these experiments incubation vessels were protected from light. In all cases total ethanol present in the medium was less than 0.1%.

Measurement of calcium uptake

Determinations of ^{45}Ca uptake were carried out using a modification of the procedure previously described by Giuliani and Boland (1984). Briefly, after treatment of preparations with $1,25(OH)_2D_3$ and/or blockers, $^{45}CaCl_2$ (2 μCi/ml) was added to the incubation medium. The uptake period was 2 min. The ventricular slices were then quickly washed with cold unlabelled medium containing the same concentration of $CaCl_2$ in order to remove ^{45}Ca non-specifically adsorbed on the membrane (Borle, 1975). The tissue was dissolved in 1 M NaOH-1% sodium dodecyl sulfate (SDS) in a boiling water bath for 30 min. Aliquots were taken for measurement of radioactivity in a liquid scintillation spectrometer and protein determination by the method of Lowry (Lowry et al., 1951).

Microsomal protein phoshorylation

Cardiac muscle microsomes were obtained by homogenization of treated and control tissue slices in a buffer containing 50 mM Tris pH 7.0, 10 mM $MgCl_2$, 0.5 mM 2*p*-methylsulfonylfluoride and 250 mM sucrose using a Ultraturrax homogenizer 5×5 s. The homogenate was centrifuged 10 min at $1025 \times g$. The supernatant was filtered through a double layer of cheesecloth. Powdered KCl was added to reach a final KCl concentration of 600 mM. The samples were incubated in an ice bath to dissolve actomyosin followed by centrifugation at $25,000 \times g$ for 30 min. Microsomes were collected by centrifugation of the supernatant at $133,000 \times g$ for 60 min. The microsomal pellet was resus-

pended with homogenization buffer and centrifuged at 133,000 × *g* for 30 min. The membranes were resuspended in a buffer containing 50 mM Tris pH 7.0. 250 mM sucrose and 0.5 mM *p*-phenylmethylsulfonyl fluoride.

Phosphorylation of microsomal membranes (300 μg) was carried out by incubation in a medium containing 50 mM Tris, pH 7.0, 10 mM $MgCl_2$ and 1 mM $CaCl_2$ (or 0.2 mM EGTA in the Ca-free phosphorylation medium) during 1 min at 30 °C. Phosphorylation was initiated by addition of [^{32}P]ATP (10 μCi/100 μl; 10 μM). After 2 min the reaction was stopped by addition of an equal volume of 2% SDS, 0.1% 2-mercaptoethanol, 10% sucrose, 10 mM bicine pH 8.3. Proteins were separated as described by Laemmli (1970). Slabs were stained with Coomassie blue. Gels were autoradiographed at −20 °C using Kodak X-Omat film. In addition, the different bands obtained were excised from the gel, dissolved in H_2O_2 at 100 °C and the radioactivity determined in a liquid scintillation spectrometer.

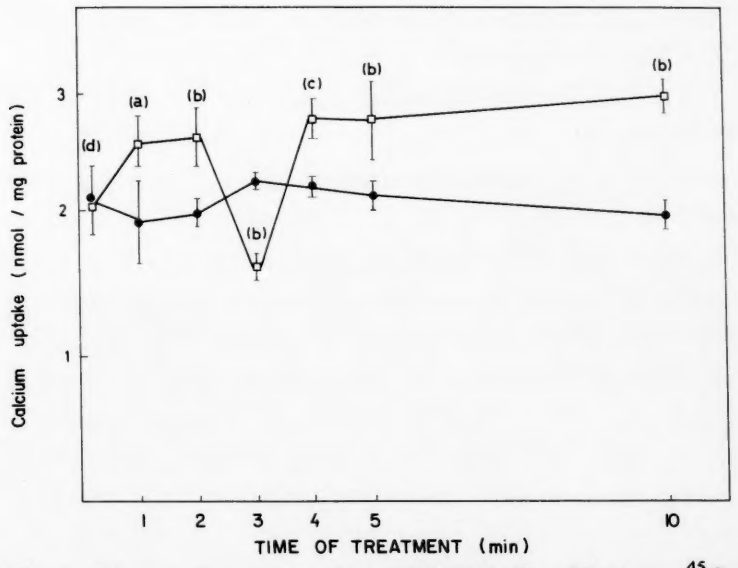

Fig. 1. Time course of in vitro 1,25(OH)$_2$D$_3$ effects on ^{45}Ca uptake by vitamin D-deficient chick cardiac muscle. Left ventricle slices were incubated 20 min in Krebs-Henseleit solution containing 0.5 mM Ca and 0.2% glucose followed by treatment with 1,25(OH)$_2$D$_3$ (1.2×10^{-10} M) for the times indicated. Ca uptake was measured for 2 min after the addition of $^{45}CaCl_2$ (2 μCi/ml). Each point represents the means ± SD; $n = 8$. (●) Control; (○) treated. [a] $p < 0.02$; [b] $p < 0.01$; [c] $p < 0.001$; [d] not significant.

Results

The rapid effects of 1,25(OH)$_2$D$_3$ on Ca uptake by cardiac muscle in vitro were studied. As shown in Fig. 1, exposure of left ventricular slices obtained from vitamin D-deficient chicks to 1.2×10^{-10} M 1,25(OH)$_2$D$_3$ for only 1 and 2 min resulted in a stimulation of ^{45}Ca uptake by the tissue (28%, $p < 0.02$, and 26%, $p < 0.01$, increase, respectively). A decrease (−27%, $p < 0.01$) was observed at 3 min. This change was followed by a stimulation of Ca uptake at 4, 5 and 10 min. Treatment of left ventricle preparations from chicks grown on a diet containing normal levels of vitamin D with 1.2×10^{-10} M 1,25(OH)$_2$D$_3$ for 5 min also significantly increased ^{45}Ca uptake (2.86 ± 0.12 vs. 3.58 ± 0.23 nmol Ca/mg protein, for control and 1,25(OH)$_2$D$_3$-treated animals, respectively).

The stimulation of ^{45}Ca uptake produced by 1,25(OH)$_2$D$_3$ after 10 min was dose-dependent. As shown in Fig. 2, hormone concentrations as low as 10^{-12} M produced a statistically significant increase of ^{45}Ca incorporation into ventricular tissue (+13%, $p < 0.05$). The heart Ca^{2+} uptake

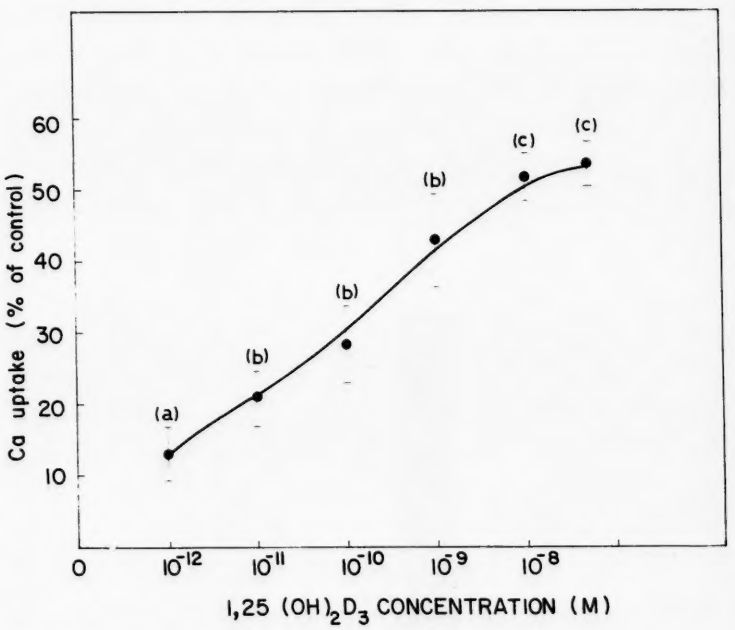

Fig. 2. Dose–response of 1,25(OH)$_2$D$_3$ rapid effects on cardiac muscle Ca uptake. Left ventricle slices from vitamin D-deficient chicks were incubated with the indicated 1,25(OH)$_2$D$_3$ concentrations or ethanol (control) for 10 min. ^{45}Ca uptake was measured as indicated in Fig. 1. Results are expressed as percent changes with respect to controls (1.98 ± 0.17 nmol/mg protein). Each point represents the means ± SD; $n = 4$. [a] $p < 0.05$; [b] $p < 0.01$; [c] $p < 0.001$.

responses increased up to 10^{-8} M $1,25(OH)_2D_3$ (+52%, $p < 0.001$).

To investigate the specificity of the effects of $1,25(OH)_2D_3$, ^{45}Ca uptake by heart muscle preparations was measured after treatment for 10 min with $1,25(OH)_2D_3$, $24,25(OH)_2D_3$, $25OHD_3$ or vitamin D_3. When sterol concentrations close to those found in chick blood total levels (Horst et al., 1982) were used, only $1,25(OH)_2D_3$ caused a significant stimulation in cardiac Ca^{2+} uptake (Fig. 3). At 20-fold higher concentrations, $24,25(OH)_2D_3$, $25OHD_3$, and vitamin D_3 to a lesser extent, also increased tissue ^{45}Ca uptake (50%, 43%, 21% and 11% for $1,25(OH)_2D_3$ $24,25(OH)_2D_3$, $25OHD_3$ and vitamin D_3, respectively).

To test the possibility that the rapid effects induced by $1,25(OH)_2D_3$ on heart Ca uptake could be due to changes in lipid metabolism, [^{3}H]glycerol incorporation into lipids of control and treated tissue was measured as described in Materials and Methods. No changes were observed in incorporation of the precursor into total lipids after a 10 min treatment interval with $1,25(OH)_2D_3$ (1035 ± 193 vs. 968 ± 88 cpm/μmol lipid P for control and treated tissue, respectively). The distribution of radioactivity in lipid fractions isolated by thin-layer chromatography showed no significant differences in labelling of phosphatidylinositol, phosphatidylserine, sphingomyelin, phosphatidylcholine, phosphatidylethanolamine and neutral lipids, between control and $1,25(OH)_2D_3$-treated samples (data not shown).

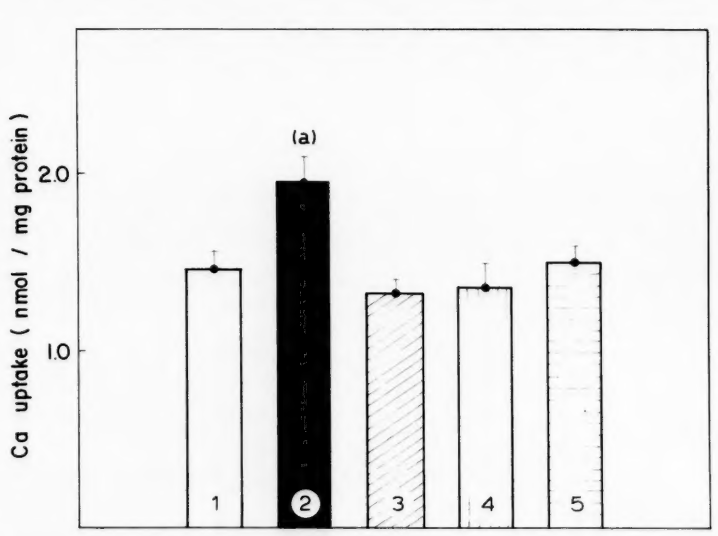

Fig. 3. Specificity of $1,25(OH)_2D_3$ rapid effects on Ca uptake by cardiac muscle. Left ventricle slices were incubated with vitamin D_3 sterols for 10 min. ^{45}Ca uptake was measured as indicated in Fig. 1. (1) Control; (2) 1.2 M $1,25(OH)_2D_3$; (3) 5.5×10^{-9} M $24,25(OH)_2D_3$; (4) 6×10^{-8} M $25OHD_3$; (5) 6×10^{-8} M vitamin D_3. $n = 5$; means ± SD are given. [a] $p < 0.01$.

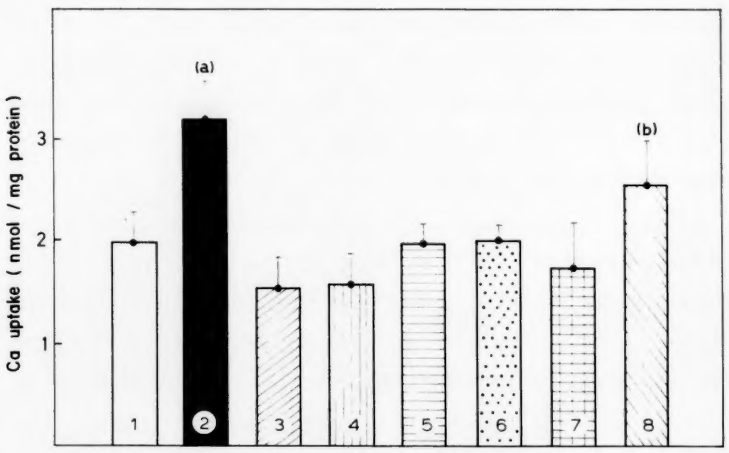

Fig. 4. Inhibition by calcium channel blockers of the $1,25(OH)_2D_3$-dependent rapid stimulation of cardiac muscle Ca uptake. Nifedipine (34 μM), verapamil (10 μM) and nitrendipine (34 μM) were added to the incubation medium 5 min prior to the addition of 1.2×10^{-10} M or ethanol. After 10 min of treatment with the metabolite, ^{45}Ca uptake was measured as indicated in Fig. 1. (1) control; (2) $1,25(OH)_2D_3$; (3) verapamil; (4) verapamil + $1,25(OH)_2D_3$; (5) nifendipine; (6) nifendipine + $1,25(OH)_2D_3$; (7) nitrendipine; (8) nitrendipine + $1,25(OH)_2D_3$, $n = 6$; means ± SD are given. [a] $p < 0.001$, compared to control; [b] $p < 0.02$ compared to + $1,25(OH)_2D_3$.

The effects of different Ca channel blockers on the rapid increase of Ca uptake by vitamin D-deficient chick cardiac muscle induced by $1,25-(OH)_2D_3$ were studied. As shown in Fig. 4, the stimulation of ^{45}Ca uptake by heart tissue caused by $1,25(OH)_2D_3$ (10 min treatment interval) could be completely abolished by nifedipine (35 μM) or verapamil (10 μM). Nitrendipine (35 μM) partially blocked the effects of the sterol. These results could be regarded as suggestive of Ca^{2+} channel involvement in the tissue response to the hormone.

Various lines of evidence have indicated that the activity of cardiac calcium channels is modulated by phosphorylation (Bkaily and Sperelakis, 1984; Reuter et al., 1986; Hosey and Lazdunski, 1988). To study whether $1,25(OH)_2D_3$ affects heart membrane protein phosphorylation, microsomes isolated from vitamin D-deficient chick left ventricle tissue treated with $1,25(OH)_2D_3$ or vehicle for 1–10 min were incubated with [^{32}P]ATP in the presence or absence of Ca^{2+} in the phosphorylation medium. Proteins were separated by

SDS-polyacrylamide gel electrophoresis (PAGE) using a polyacrylamide gradient of 10–20%. Two major radioactive bands corresponding to relative molecular masses of 43 kDa and 55 kDa were detected by autoradiography. After a 10 min treatment interval with $1,25(OH)_2D_3$, a stimulation of phosphorylation of the 43 kDa protein and to a lesser extent of the 55 kDa protein was observed, both in the presence and absence of Ca in the reaction medium (Fig. 5). However, labelling of the macromolecules was greatly increased when Ca^{2+} was omitted. The latter may possibly reflect a decrease in Ca-dependent phosphatase activity. Table 1 provides a quantitative description of changes in phosphorylation of the 55 and 43 kDa protein fractions caused by $1,25(OH)_2D_3$ at various treatment intervals. Higher levels of phosphorylation were measured in the 43 kDa than in the 55 kDa protein at all treatment intervals. The stimulation of phosphorylation of both macromolecules was detected as fast as at 1 min and maximum responses were detected at 2 min (+73%) and 3 min (+54%) for the 55 kDa and 43 kDa proteins, respectively.

TABLE 1

TIME-COURSE OF $1,25(OH)_2D_3$-INDUCED CHANGES IN PHOSPHORYLATION OF 55 kDa AND 43 kDa CARDIAC MICROSOMAL PROTEINS

The tissue was treated with 1.2×10^{-10} M $1,25(OH)_2D_3$ or vehicle for 1–10 min. Microsomes were isolated and phosphorylated with [γ-^{32}P]ATP as indicated in Fig. 5. Proteins were separated by SDS-PAGE. Gels were sliced (2 mm) and the radioactivity was counted. Results of a typical experiment are shown.

Time of treatment (min)	Protein phosphorylation (cpm)			
	55 kDa		43 kDa	
	Control	$1,25(OH)_2D_3$	Control	$1,25(OH)_2D_3$
1	168	191 (+14%)	1,485	2,000 (+35%)
2	172	298 (+73%)	3,084	4,273 (+39%)
3	176	258 (+47%)	2,666	4,105 (+54%)
10	255	336 (+32%)	2,301	3,334 (+45%)

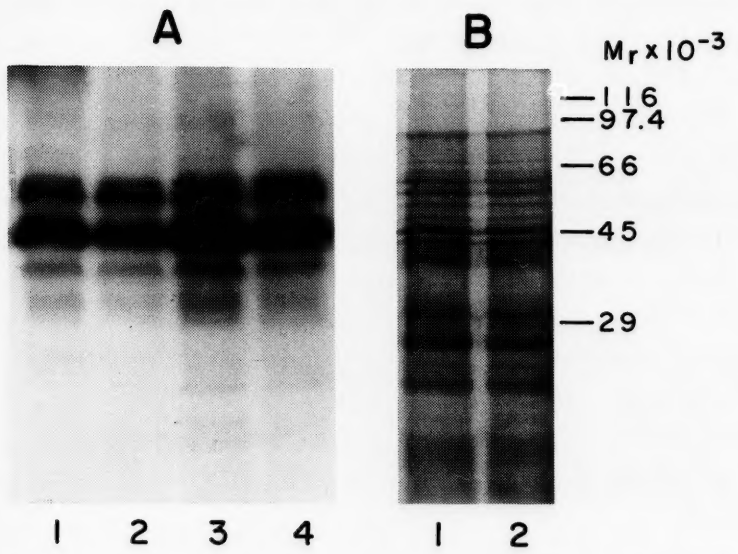

Fig. 5. $1,25(OH)_2D_3$ stimulation of cardiac microsomal protein phosphorylation. Chick cardiac muscle was treated with 1.2×10^{-10} M $1,25(OH)_2D_3$ or ethanol for 10 min. After tissue homogenization microsomes were isolated by differential centrifugation as indicated in Materials and Methods. Microsomal proteins were phosphorylated with [^{32}P]ATP in the presence and absence (+0.2 mM EGTA) of 1 mM Ca^{2+}. Proteins were separated by SDS-PAGE using a 10–20% polyacrylamide gradient. Gels were autoradiographed (*A*) and stained with Coomassie blue (*B*). Lane 1: treated, +Ca; lane 2: control, +Ca; lane 3: treated, −Ca; lane 4: control, −Ca.

Discussion

The results of the present investigations showed that $1,25(OH)_2D_3$ has the ability to stimulate within 1–10 min ^{45}Ca uptake by vitamin D-deficient chick cardiac muscle in vitro. The hormone acted in a dose-dependent fashion and its effects could be detected at physiologically relevant doses (10^{-12} to 10^{-11} M). The fast action of $1,25(OH)_2D_3$ on heart muscle Ca uptake exhibited specificity as $24,25(OH)_2D_3$ and $25OHD_3$, other major vitamin D_3 metabolites, and vitamin D_3 itself induced weaker changes only at considerably higher concentrations. An interesting feature of the time-course of modifications in ^{45}Ca uptake by cardiac muscle caused by $1,25(OH)_2D_3$ was the transient decrease in tissue labelling at 3 min which occurred after the stimulation at 1–2 min. This change was consistently observed throughout the experiments. It may reflect increased Ca efflux from heart tissue following an initial elevation of Ca influx and/or Ca release from intracellular stores by the sterol. This interpretation is suggested by preliminary experiments in which ventricular slices were prelabelled with ^{45}Ca for 30 min and then washed with cold unlabelled medium followed by treatment with $1,25(OH)_2D_3$ for 2, 3

and 5 min. Stimulation of ^{45}Ca release into the medium was observed only after the 3 min treatment interval (5 ± 0.45 nmol Ca/mg protein vs. 6 ± 0.22 nmol Ca/mg protein, for control and treated preparations, respectively; $p < 0.01$). However, further experimental work is required in order to further test this hypothesis.

Due to the short times needed to produce the tissue responses (1–10 min), it is unlikely that the effects of $1,25(OH)_2D_3$ on cardiac muscle Ca uptake were mediated by de novo RNA or protein synthesis. Alterations in membrane lipids have been previously involved in the stimulation of enterocyte ion transport by the hormone (Fontaine et al., 1981; Drueke et al., 1985). However, in the present study the lack of changes in [^{3}H]glycerol incorporation into heart muscle lipids after short treatment intervals with $1,25(OH)_2D_3$ excludes the possibility that modifications in lipid synthesis mediate the rapid action of the sterol on Ca uptake by heart tissue. Similar observations have been made in skeletal muscle (de Boland and Boland, 1987).

The observation that the rapid stimulatory effect of $1,25(OH)_2D_3$ on heart Ca uptake could be inhibited by verapamil, nifendipine and partially by nitrendipine indicates that the action of the sterol is mediated by the activation of voltage-dependent Ca channels. Similar lines of evidence have involved $1,25(OH)_2D_3$ in the modulation of Ca channel activity in skeletal muscle (de Boland and Boland, 1987) and intestine (de Boland et al., 1989). Furthermore, single-channel recordings by means of the patch-clamp technique have shown that $1,25(OH)_2D_3$ affects dihydropyridine-sensitive calcium currents in clonal rat osteosarcoma cells (Caffrey and Farach-Carson, 1989).

We have found that incubation of the in vitro heart preparations with $1,25(OH)_2D_3$ for intervals similar to those at which the rapid Ca uptake response to the hormone is elicited stimulates the phosphorylation of at least two proteins of 43 kDa and 55 kDa in isolated microsomal membranes. These results may bear a clue on the mechanism involved in the fast action of $1,25(OH)_2D_3$ on cardiac muscle Ca uptake. Membrane protein phosphorylation is a key metabolic event participating in the regulation of cardiac calcium channels (Bkaily and Sperelakis, 1984; Flockerzi et al., 1986; Reuter et al., 1986). Interesting enough, the presence of a peptide component of 55–54 kDa in the purified heart Ca channel complex which binds dihydropyridines and is phosphorylated by cyclic AMP-dependent protein kinase has been reported (Rengasamy et al., 1985; Flockerzi et al., 1986). The effects of $1,25(OH)_2D_3$ on cardiac membrane protein phosphorylation could be observed in the absence of Ca^{2+} (+EGTA) in the medium in which isolated membranes were phosphorylated with [^{32}P]ATP, suggesting that they are not related to changes in activity of membrane-bound Ca^{2+}/calmodulin- or Ca^{2+}/phospholipid-dependent protein kinases. Evidence has been obtained indicating that cAMP-dependent protein kinase mediates $1,25(OH)_2D_3$ activation of voltage-operated Ca channels in muscle (Fernandez et al., 1990) and intestine (de Boland and Norman, 1990). Moreover, recent experimental work of the laboratory employing cultured chick cardiac cells has shown a suppression of the fast $1,25(OH)_2D_3$-induced changes in Ca uptake by protein kinase A inhibitors (J. Selles and R. Boland, unpublished observations). However, additional studies are required to establish whether such a mechanism operates in heart tissue.

Finally, the results of this study and of previous investigations (Walters et al., 1987) suggest that $1,25(OH)_2D_3$ exerts a direct action in regulating intracellular Ca^{2+} in heart muscle. This role may be important in understanding certain aspects of cardiovascular dysfunction associated to vitamin D deficiency (Weishaar and Simpson, 1987, 1989; Hochhauser et al., 1988) and renal failure (Coratelli et al., 1984; McGonigle et al., 1984).

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Effects of in vivo dihydrotestosterone treatment on changes in nocturnal surge of prolactin, luteal ultrastructure and *P*-450$_{scc}$ mRNA and protein content in pregnant rats

R. Sridaran [1], Carol J. Smith [1] and JoAnne S. Richards [2]

[1] *Department of Physiology, Morehouse School of Medicine, Atlanta, GA 30310-1495, U.S.A., and* [2] *Department of Cell Biology, Baylor College of Medicine, Houston, TX 77030, U.S.A.*

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Summary

We have demonstrated previously that the administration of dihydrotestosterone (DHT) decreases plasma progesterone levels within 24 h and thus, results in abortion during the first half of pregnancy (Am. J. Physiol. 241 (1981) E444–E448). The purpose of this study was to determine (a) if the administration of DHT suppresses plasma prolactin levels or its nocturnal surge within 24 h after the treatment, (b) how soon after the commencement of treatment do the concentrations of DHT increase and progesterone levels decrease in the circulation, (c) the ultrastructural changes that occur in corpora lutea, and (d) the changes in luteal *P*-450 side-chain cleavage (*P*-450$_{scc}$) enzyme and mRNA content upon DHT treatment. Within 24 h after the commencement of DHT treatment, the nocturnal surge of prolactin, detected in both groups on day 10 at 03.30 h, was inhibited in DHT-treated rats as compared to controls. The non-surge levels of prolactin at 05.00 and 06.00 h were not different between groups. The intraovarian DHT pellet increased plasma levels of the steroid 3-fold within 2 h (blood samples were taken at 2-hourly intervals) when compared to controls. By 24 h DHT levels were decreased but were still higher than controls. Plasma progesterone levels began to fall 6 h after the commencement of treatment. Luteal tissue from animals treated with DHT appeared steroidogenic, and contained more lipid droplets than controls. No changes in mitochondrial structure or number were observed. Although the amount of luteal *P*-450$_{scc}$ mRNA was lower in the DHT-treated rats compared to control rats, this decrease was not associated with a decline in *P*-450$_{scc}$ protein content within 24 h after DHT treatment. These data suggest that exogenous administration of DHT in pregnant rats results in acute high levels of plasma DHT leading to decreased plasma progesterone levels. The decline in plasma progesterone may be due to decreased cholesterol transport to

Address for correspondence: Dr. R. Sridaran, Department of Physiology, Morehouse School of Medicine, 720 Westview Drive, S.W., Atlanta, GA 30310-1495, U.S.A.

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the mitochondria or to decreased esterase activity. The lower progesterone levels, in turn, may lead to inhibition of the nocturnal prolactin surge. This study provides new information and insight into the abortifacient mechanism of DHT.

Introduction

Dihydrotestosterone (DHT) has been known to have antifertility effects in both sexes of several species. Plasma concentrations of DHT are significantly higher in non-pregnant women with recurrent pregnancy disorders of unknown etiology, than the levels found in non-pregnant normal women (Tajic et al., 1981). Administration of DHT on the day of metestrus in mice results in a significant reduction in the number of females becoming pregnant and bearing normal fetuses (Nandedkar et al., 1981). DHT also causes follicular atresia in female rats (Nandedkar, 1981). Thus, if the synthesis and/or release of DHT is increased, it has profound antifertility effects.

Our laboratory has been interested in determining the mechanism of action of DHT in terminating pregnancy when administered exogenously. Our previous studies have indicated that DHT treatment results in abortion when administered during early pregnancy in the rat (Sridaran and Gibori, 1981). Administration of DHT on day 9 of pregnancy suppresses serum progesterone by 50% within 24 h, after which progesterone levels remain low through day 12; the fetuses are alive on day 12 but complete abortion occurs by day 15.

Because serum progesterone levels fall prior to fetal death, we conclude that the effect of DHT is not directly detrimental to the fetuses; instead, the dramatic fall in serum progesterone causes the abortion. At the time DHT-treated rats have completely aborted, estradiol levels in the ovarian vein plasma are not different from controls indicating that the abortifacient effect of DHT is not mediated by estradiol.

The high levels of progesterone needed to maintain pregnancy are dependent on the action of several other hormones, including prolactin and other steroid hormones. During early pregnancy, luteinizing hormone (LH) stimulates the synthesis of testosterone in the corpus luteum (Sridaran and Gibori, 1983) which is converted to estradiol by the aromatase enzyme system (Elbaum and Keyes, 1976; Gibori et al., 1982). Prolactin maintains receptors for estradiol in the ovary (Gibori et al., 1979). Estradiol coupled to its receptor enhances luteal progesterone synthesis by increasing the availability of cholesterol (Gibori et al., 1984; Khan et al., 1984, 1985). Since DHT treatment suppresses luteal progesterone synthesis (Sridaran and Gibori, 1981) it may result in other effects on the corpus luteum which can be examined by studying its ultrastructural and *P*-450 side-chain cleavage ($P\text{-}450_{scc}$) enzyme and mRNA content in corpora lutea after in vivo treatment with DHT to more precisely define the intracellular events that lead to decreased luteal progesterone production.

Prolactin exhibits diurnal or nocturnal surges daily until day 9 and 11 of pregnancy, respectively (Smith and Neill, 1976). Therefore, one-point serum prolactin determination daily may not show the effect of DHT on the secretion of prolactin. Steroids are known to modulate these surges. Therefore, we sought to determine the time sequence of increased plasma DHT concentration, the fall in plasma progesterone levels and changes in the prolactin surges. Further we sought to determine the effects of DHT on luteal cell structure (mitochondrial morphology) and function, namely changes in $P\text{-}450_{scc}$ enzyme and mRNA content.

Materials and methods

Materials

Iodine-125 for prolactin iodination was supplied as sodium iodide in dilute sodium hydroxide solution at pH 7–11, free from reducing agents, by Amersham Corp. (Arlington Heights, IL, U.S.A.). [^{3}H]Progesterone and [^{3}H]dihydrotestosterone were purchased from NEN Research Products (Boston, MA, U.S.A.). DHT (5α-androstan-17β-ol-3-one) and heparin, sodium salt were obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Ether for anesthesia was from Mallinckrodt (Paris, KY, U.S.A.), hexanes, spectranalized was from

Fisher Scientific Co. (Norcross, GA, U.S.A.), ethyl acetate, photrex was from J.T. Baker Chemical Co. (Phillipsburg, NJ, U.S.A.), Polybed 812 was from Polysciences (Warrington, PA, U.S.A.), and ethanol, 200 proof was from Pharmco (Philadelphia, PA, U.S.A.). The following reagents were purchased from Ted Pella (Tustin, CA, U.S.A.): lead citrate, paraformaldehyde, uranyl acetate and 200 mesh copper grids. Glutaraldehyde, cacodylate, propylene oxide and OsO_4 were obtained from Electron Microscopy Sciences (Fort Washington, PA, U.S.A.). All other reagents were of the highest available grade of purity.

Animals

Pregnant rats of Sprague-Dawley strain were purchased from the Holtzman Co. (Madison, WI, U.S.A.). They were housed one per cage with a 14 h photoperiod (lights on at 05.00, off at 19.00 h) in a temperature controlled room at 24–26 °C. Purina rat chow (Ralston-Purina, St. Louis, MO, U.S.A.) and water were provided ad libitum. The day of insemination was designated as day 1 of pregnancy.

Hormonal treatment and surgery

DHT pellets were prepared by tamping the crystals into the cylindrical end of a pellet maker designed to make a pellet of a given weight per unit of length (Shelesnyak and Berenblum, 1952). On day 9 of pregnancy, laparotomy was performed under ether anesthesia in rats to observe the implantation sites. A silastic cannula was inserted into the right atrium via the external jugular vein in each rat (Terkel, 1972). One DHT pellet weighing about 4 mg was implanted under each ovarian bursa. DHT insertion was completed by 12.00 h colony time. Ovarian bursas were slightly cut in control. In the first experiment, blood samples (350 μl) were collected into heparinized tubes at 03.30, 05.00 or 06.00 h of colony time on day 10. In the second experiment, blood samples (300 μl) were collected into heparinized tubes at 2-hourly intervals for 6 h on day 9 and once on day 10 to determine the profiles of progesterone and DHT in the circulation. An equal volume of heparinized saline (100 IU/ml) was injected after each bleeding. Plasma was stored frozen at −20 °C.

Selection of corpora lutea

On day 10 of pregnancy, the ovaries were dissected out from the rats while the rats were under ether anesthesia. The corpora lutea were separated, and cleaned of adhering follicles under a dissecting microscope. The morphological and *P*-450_{scc} studies were carried out only on the largest size of corpora lutea that were present in the ovary. These corpora lutea of pregnancy were pinkish in color in untreated controls while they were pale and fragile in DHT-treated rats. Most of the time, the number of these corpora lutea and the number of implantation sites were identical in an individual rat in both groups.

Electron microscopy

Corpora lutea were obtained from eight different rats on day 10, four controls and four DHT-treated animals. The tissues were fixed overnight in a 2.5% glutaraldehyde/2% paraformaldehyde solution buffered with 0.1% cacodylate at pH 7.4. The tissues were post-fixed in 2% OsO_4 in cacodylate. After washing, the tissues were stained en bloc in saturated aqueous uranyl acetate, then dehydrated with ethanol. After infiltration with propylene oxide followed by Polybed 812, the tissues were embedded in Polybed 812.

A Porter-Blum MT2B ultramicrotome was used to section the tissue. The sections were placed on 200 mesh copper grids, stained with uranyl acetate followed by lead citrate, and examined in a JEOL JEM 1200 EX transmission electron microscope.

Lipid droplets were quantified using a stereological technique described by Weibel (1969). A grid approximately 75 × 90 mm was placed randomly on the material (magnified 8000 × 80 kV), and the number of lipid droplets enclosed within this area was quantified. Twelve sections from two different areas of each tissue were examined.

Radioimmunoassays

Plasma concentrations of progesterone were measured after hexane extraction using the procedure described by Gibori et al. (1977). This assay employs a specific antibody (GDN-337) prepared against progesterone provided by Dr. G.D. Niswender. The specificity of the antiserum is very high. The sensitivity of the assay was 0.10 ng/assay tube.

Plasma concentrations of DHT were measured after ethyl acetate/hexane (3 : 2) extraction by a radioimmunoassay using a highly specific antiserum (Radioassay System Laboratories, Carson, CA, U.S.A.; catalog 155; lot r-140). The antiserum crossreacts 8% with testosterone and none with estradiol, progesterone and corticosterone. The specificity, validity and reliability of this assay have been previously reported by us (Sridaran and Gibori, 1981). The standard curve ranged from 0.005 to 1.0 ng and all working standards were in a volume of 0.5 ml. The sensitivity of the assay was 10 pg/assay tube. The coefficient of variation for intraassay was 11.6% and that for interassay was 19.3%.

Plasma prolactin and LH were measured by using the kits obtained from National Hormone and Pituitary Program, NIDDK, following the procedures described in the kits. Antirat PRL (rPRL-S9) was used at an initial dilution of 1 : 2500. NIDDK-rPRL-RP-2 and NIDDK-rPRL-RP-3 are equal in potency. NIDDK-rPRL-RP-3, which has a biological potency equivalent to 2.8 × NIDDK-rPRL-RP-1, was used as a standard. rPRL antigen (NIDDK-rPRL-I-5) was iodinated using the chloramine-T method. The sensitivity of the assay was 0.03 ng/tube. Anti-rat LH serum was used at an initial dilution of 1 : 30,000. NIDDK-rLH-RP-2 which has a biological potency equivalent to 61 × NIDDK-rLH-RP-1 was used as a standard. rLH antigen (NIDDK-rLH-I-6) was iodinated by chloramine-T method. The sensitivity of the assay was 0.03 ng/ml.

P-450$_{scc}$

Immunoblot analysis. The procedure for tissue homogenization, detergent (CHAPS) solubilization of membrane proteins, sodium dodecyl sulfate-polyacrylamide gel electrophoresis and immunoblot analysis of *P*-450$_{scc}$ protein was as described previously (Goldring et al., 1987; Hedin et al., 1987; Hickey et al., 1988). The content of *P*-450$_{scc}$ enzyme was quantified using gel transfer and immunoblotting procedures by subjecting equal amounts of protein (200 μg) as previously described (Hedin et al., 1987). The affinity-purified rabbit anti-rat *P*-450$_{scc}$ antiserum was used in the immunoblot analysis at 1 : 50 dilution (Farkash et al., 1986) along with [^{125}I]iodoprotein-A (ICN; 1×10^6 cpm/ml) to visualize the rat luteal *P*-450$_{scc}$ enzyme. Relative changes in *P*-450$_{scc}$ content were quantified by counting the ^{125}I-labelled *P*-450$_{scc}$ bands in a gamma counter.

Northern blot analysis. Total RNA was extracted in 4 M guanidine isothiocyanate and collected by CsCl density centrifugation (Chirgwin et al., 1979). The RNA was phenol/chloroform extracted, ethanol precipitated, resuspended in autoclaved water, stored at −70 °C, and measured by absorbance at A_{260}. 20 μg total RNA were denatured in 45% formamide/5.4% formaldehyde for 15 min at 50 °C and electrophoresed at 4 °C in a 1.5% agarose gel containing 1.0 formaldehyde and 20 mM sodium phosphate, pH 7.0 in the presence of an RNA standard ladder. Gels were stained in acridine orange and photographed. RNA was transferred to 1.2 μm BioDyne filters (ICN), prehybridized, hybridized to a nick-translated 1.2 kb *P*-450$_{scc}$ cDNA probe (1×10^6 cpm/ml), and washed according to the specifications of ICN (Goldring et al., 1987). Northern blot transfers reveal a single *P*-450$_{scc}$ mRNA species about 2.0 kb in size (Goldring et al., 1987). The relative amounts of *P*-450$_{scc}$ mRNA in control and treated groups were determined by densitometric scanning of autoradiographs.

Statistics

Differences between groups were analyzed by one-way analysis of variance, followed by the Neuman-Keuls test when differences were significant. Student's *t*-test was utilized to compare differences in results from controls and DHT-treated rats when only single-point determinations were made. Probability (p) values of less than 0.05 were considered statistically significant.

Results

The nocturnal surge of plasma prolactin (Fig. 1) was detected at 03.30 h in the control and DHT-treated rats within 24 h after treatment. However, the magnitude of nocturnal surge of prolactin decreased dramatically after DHT treatment as compared to control values. The non-surge levels, determined at 05.00 and 06.00 h, were not different from control values. Plasma LH levels were not altered by the treatment at 03.30 h

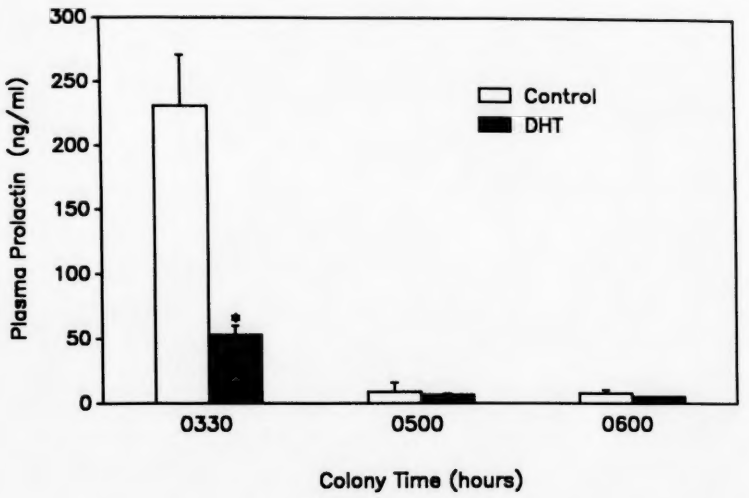

Fig. 1. Plasma levels of prolactin on day 10 of pregnancy. A silastic cannula was inserted into the right atrium via the external jugular vein in each rat under ether anesthesia on day 9 prior to implanting dihydrotestosterone pellet under each ovarian bursa, as described in Materials and Methods. Ovarian bursas were slightly cut in control. Blood samples (350 μl) were collected at the time shown on the x-axis. Each bar represents the mean $\pm$SEM ($n = 5$–6 rats). $^{*}p < 0.05$ with respect to control.

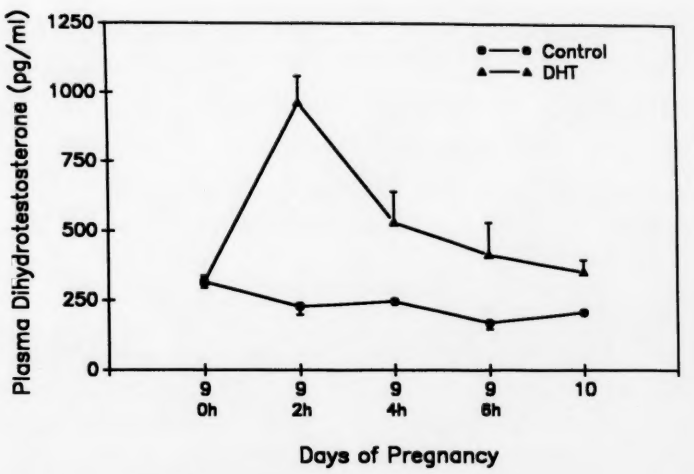

Fig. 3. Plasma dihydrotestosterone levels. Treatment and collection of blood samples are as described in the legend to Fig. 2. Each bar represents the mean $\pm$ SEM ($n = 4$–12 rats).

(control: 583 ± 123 vs. DHT: 406 ± 127 pg/ml) or at 06.00 h (control: 350 ± 55 vs. DHT: 389 ± 122 pg/ml).

Plasma progesterone concentrations (Fig. 2) began to fall 6 h after the commencement of DHT treatment on day 9 and remained low through the end of the experiment on day 10. The treatment within 2 h after its commencement increased plasma levels of DHT (Fig. 3) by 3-fold when compared to controls. DHT levels later decreased

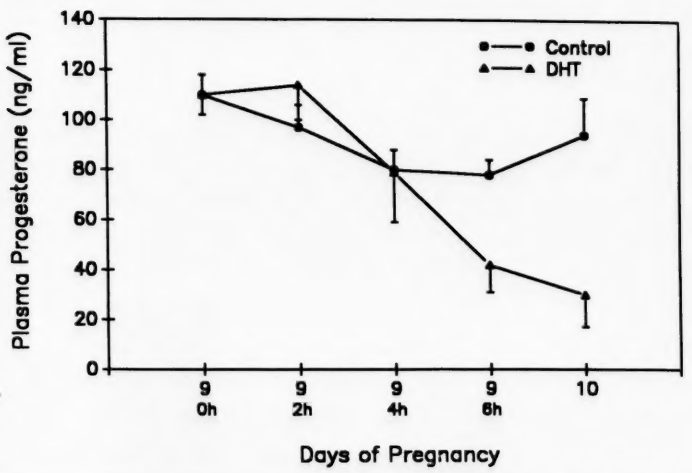

Fig. 2. Plasma progesterone levels. Rats were fitted with an indwelling intra-atrial cannula individually and treated with DHT as described in the legend to Fig. 1. Two-hourly blood samples (300 μl) were collected on day 9 with samples at 0 h representing pretreatment levels; a single sample was obtained on day 10. Each point represents the mean $\pm$ SEM ($n = 4$–12 rats).

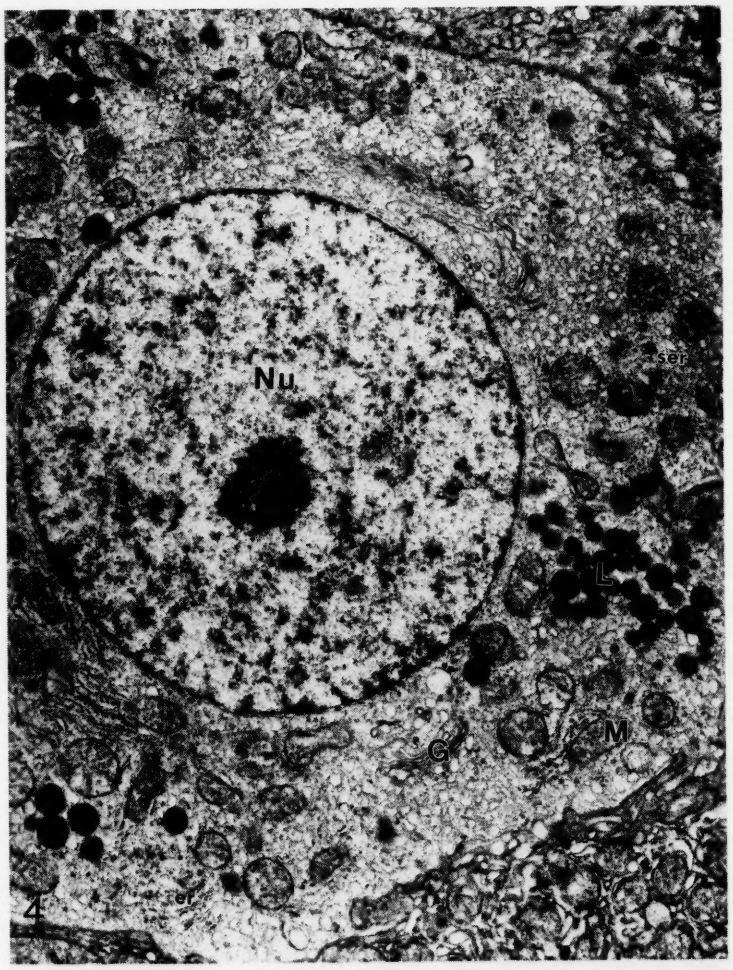

Fig. 4. Electron micrograph of the corpus luteum of a rat treated with dihydrotestosterone ($\times$12,000). Nu, nucleus; ser, smooth endoplasmic reticulum; M, mitochondria; L, lipid droplets; G, Golgi complex; er, rough endoplasmic reticulum.

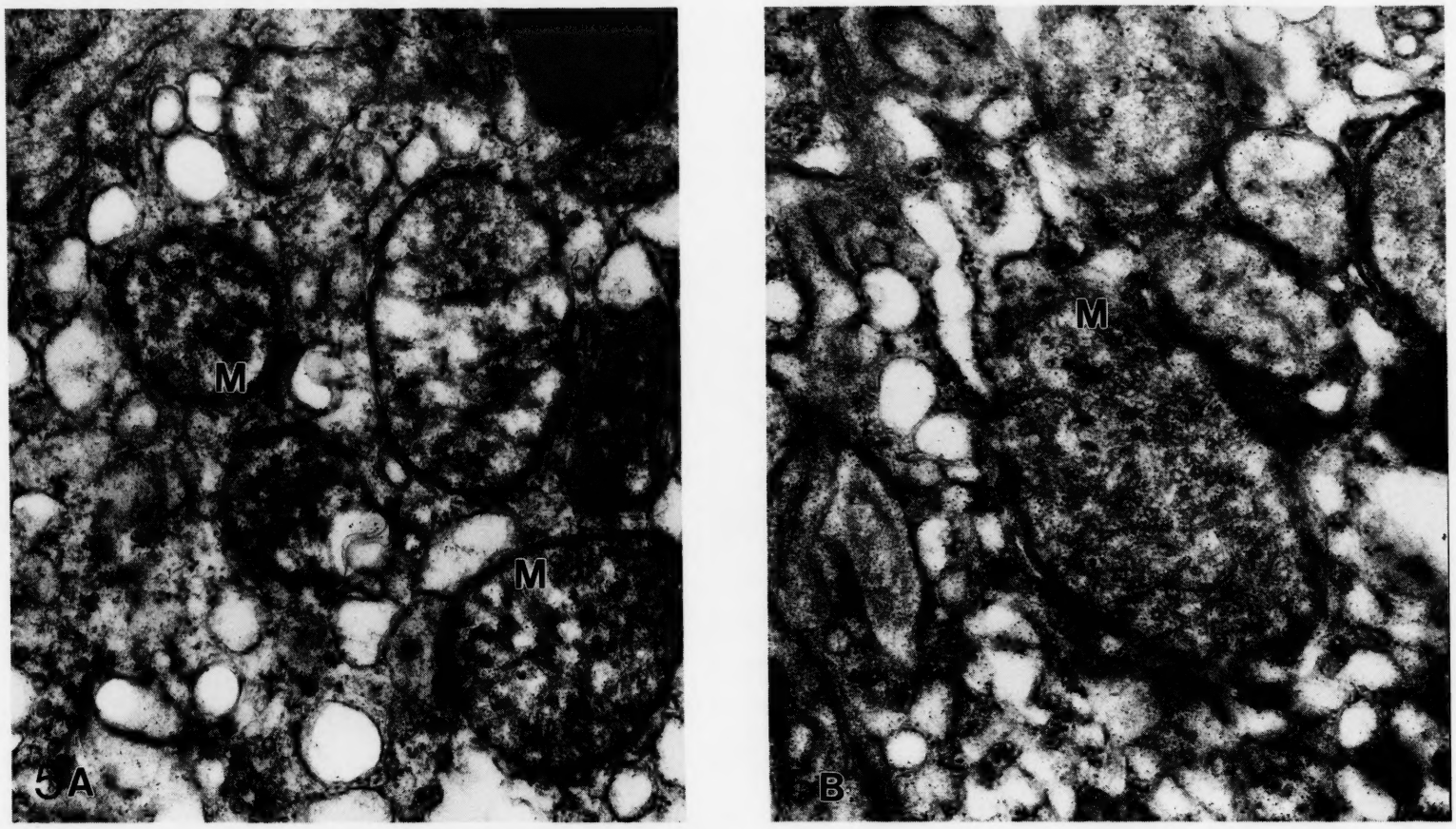

Fig. 5. Electron micrographs of the corpus luteum of a rat treated with dihydrotestosterone (×52,000; *A*) and control rat (×60,000; *B*). M, mitochondria with tubular cristae.

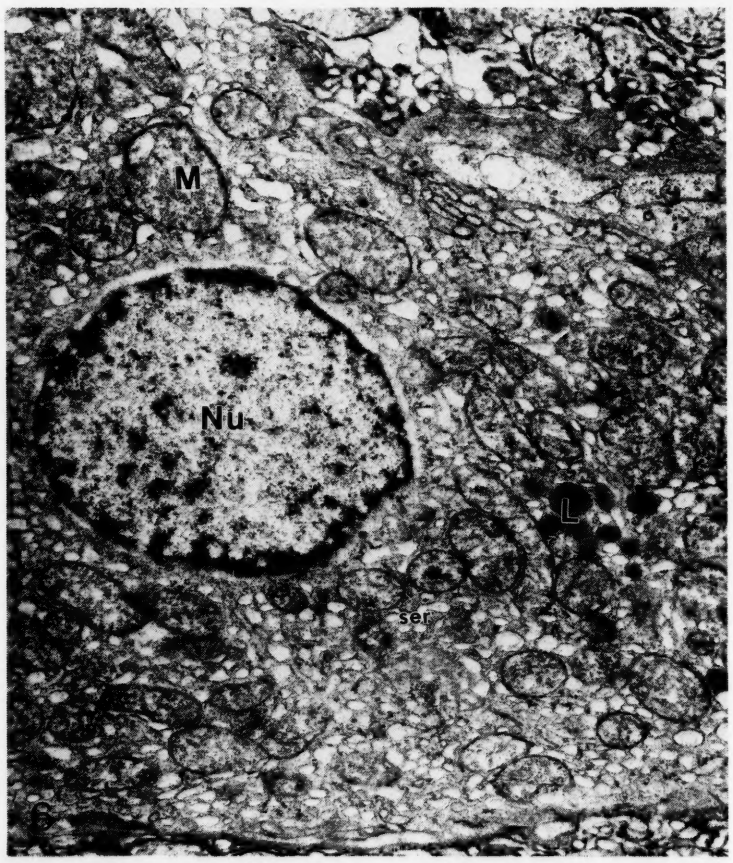

Fig. 6. Electron micrograph of the corpus luteum in a control rat (×14,000). Nu, nucleus; ser, smooth endoplasmic reticulum; M, mitochondria; L, lipid droplets.

to 354 ± 44 pg/ml by 24 h but were still higher than controls at 203 ± 10 pg/ml.

Luteal tissue from rats treated with DHT appeared steroidogenic, containing much smooth endoplasmic reticulum and numerous mitochondria with tubular cristae (Figs. 4 and 5*A*). These two organelles appeared similar in number and size to those found in tissue from control animals (Figs. 5*B* and 6). Golgi complexes, rough endoplasmic

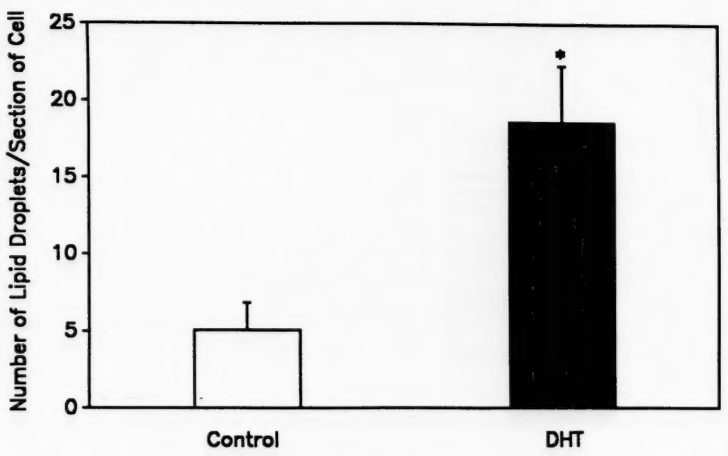

Fig. 7. Quantification of lipid droplets in the luteal tissue. Shown are the mean ± SEM.

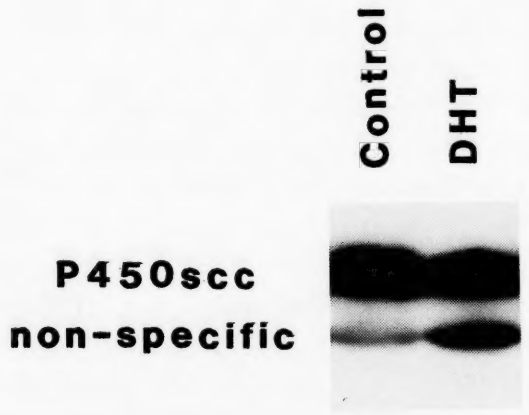

Fig. 8. Immunoblots representing $P\text{-}450_{scc}$ enzyme content in the corpora lutea. 200 μg of solubilized cell extract protein were loaded in each lane.

reticulum and polyribosomes were also present in tissue from control animals, and DHT-treated rats. The major ultrastructural change that was obvious in the luteal tissue obtained from animals treated with DHT as compared to controls was the quantity and density of lipid droplets. In comparison to tissue from the control animals, more lipid droplets were present in the tissue from DHT-treated rats (Fig. 7). The lipid droplets in tissue from DHT-treated rats were more electron-dense as compared to controls, even though all processing occurred simultaneously (Figs. 4 and 6).

The DHT treatment does not appear to have any major suppressive effect on the luteal $P\text{-}450_{scc}$ enzyme content as measured by Western blot analysis (Fig. 8; 41,011 cpm/band in controls vs. 37,849 cpm/band in DHT-treated rats). Although the amount of $P\text{-}450_{scc}$ mRNA was lower in the DHT-treated rats (relative peak area 3.1) compared to control rats (relative peak area 4.7) (Fig. 9), this decrease was not associated with a decline in $P\text{-}450_{scc}$ protein content during the 24 h time course of the experiment. Thus, changes in luteal cell content of the enzyme cannot account for the more pronounced decline in plasma levels of progesterone (Fig. 2).

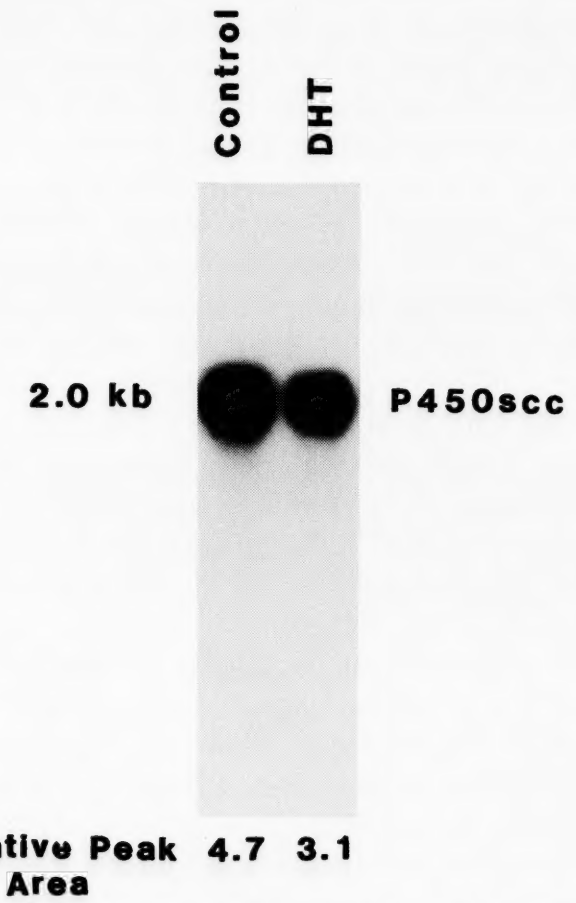

Fig. 9. Northern blots representing $P\text{-}450_{scc}$ mRNA in the corpora lutea. 20 μg of total RNA were added in each lane. Relative peak area for each lane was determined by densitometric scanning.

Discussion

Administration of DHT results in an acute rise in the plasma concentrations of DHT, followed by a decrease in plasma progesterone levels that occur prior to the nocturnal surge of prolactin in these rats. Therefore, these results support the hypothesis that the fall in plasma progesterone levels may prevent the nocturnal surge of prolactin without altering the timing of its surge. Specifically, our data demonstrate that DHT treatment inhibits the nocturnal surge of prolactin within 24 h after the commencement of the treatment, despite the fact that in these rats it occurs at the same time (around 03.30 h colony time) as in control rats. However, non-surge plasma levels of prolactin are not affected by DHT treatment. This is in agreement with the observation made by Brann et al. (1989) that in immature rats DHT has no effect on basal serum levels.

Ovarian steroids are known to modulate prolactin surges in rats; elevated progesterone levels accentuate the nocturnal surge, while elevated estradiol levels accentuate the diurnal surge and inhibit the nocturnal surge (Freeman and Sterman, 1978). The decrease in plasma progesterone levels in DHT-treated rats causes the decrease in nocturnal prolactin surge levels with no effect on

its timing. It is unlikely that the plasma estradiol levels in these rats have any detrimental effect on the nocturnal surge of prolactin because we (Sridaran and Gibori, 1981) have demonstrated that estradiol levels in the ovarian vein plasma of DHT-treated rats were not different from controls. Since the non-surge levels of prolactin in the DHT-treated rats are not different from controls, it is not expected that the treatment has any adverse effect on the luteal estradiol receptors. However, this aspect needs to be investigated further. Long-term treatment of DHT has been shown to prevent the stimulatory effect of estradiol on pituitary prolactin mRNA levels and pituitary prolactin content in intact females (Tong et al., 1989). The inhibition of estrogen-induced prolactin release by DHT requires estrogen-priming and has no effect on basal serum levels in rats (Brann et al., 1989). These observations support the hypothesis that the inhibitory effect of DHT on the nocturnal surge of prolactin is not mediated by its direct action on the pituitary. This conclusion is further strengthened by our observation that plasma LH levels are not altered by DHT treatment.

The ultrastructural details of the corpus luteum indicate that the quantity of lipid droplets is increased in DHT-treated rats. This suggests that the disruption of luteal progesterone production by DHT is after the point of cholesterol synthesis or its uptake in the biosynthetic pathway. Therefore, DHT treatment may decrease enzymatic conversion of cholesterol esters to free cholesterol, or decrease the transport of cholesterol to mitochondria. This effect of DHT appears specific and not mediated by an estradiol pathway for the following reasons. It has been demonstrated that only at elevated levels (i.e., greater than 100 μg/day s.c. treatment) does DHT bind to the estrogen receptor, translocates, and initiates an estrogenic response (Rochefort and Garcia, 1976). Luteal synthesis and release of estradiol in DHT-treated rats were normal (Sridaran and Gibori, 1981). Estradiol is known to enhance not inhibit production by increasing the availability of cholesterol (Gibori et al., 1984; Khan et al., 1984, 1985). Collectively, these data indicate that DHT treatment is not acting via an estrogenic response system and may disrupt the luteal production of progesterone by decreasing the transport of cholesterol to mitochondria.

It is also possible that treatment with DHT decreases $P\text{-}450_{scc}$ activity, an enzyme complex that is rate-limiting for steroidogenesis (McIntosh et al., 1971; Arthur and Boyd, 1974). However, this is not probable because the appearance of tubular cristae in mitochondria is remarkably similar in treated as well as in control rats and the luteal $P\text{-}450_{scc}$ enzyme has not been altered by DHT treatment. However, DHT treatment lowers the amount of $P\text{-}450_{scc}$ mRNA. Since this decrease was not associated with a decline in $P\text{-}450_{scc}$ protein, the changes in luteal cell content of the enzyme cannot be responsible for the dramatic decline in plasma progesterone levels. The $P\text{-}450_{scc}$ complex is located in the inner mitochondrial membrane (Hall, 1984), and the changes in the cristae structure may correspond to changes in $P\text{-}450_{scc}$ activity.

In another study, when rats were treated with an agonist for gonadotropin-releasing hormone (GnRH-Ag) on day 7 or 11 of pregnancy, we have demonstrated that the treatment not only decreases luteal progesterone production and increases the number of lipid droplets within 24 h after the treatment, but it also decreases the number of tubular cristae and increases the number of lamellar cristae in mitochondria (Smith et al., 1991). Rennels (1966) reported that the luteal cells obtained from hypophysectomized rats possess a greater quantity of lipid and mitochondria with lamellar cristae when progesterone production is decreased, as compared to stimulated rat luteal cells. The results of these two studies indicate that the change in mitochondrial cristae is a specific result of GnRH-Ag treatment or hypophysectomy and not a generalized mechanism which decreases progesterone production. Unlike the results obtained from hypophysectomized rats, no changes were observed in smooth endoplasmic reticulum after treatment with DHT or GnRH-Ag. One explanation is that the smooth endoplasmic reticulum may still be synthesizing cholesterol de novo. Also, enzymes necessary for further conversion of progesterone to androgens and estradiol are present in the microsomal compartment (Hall, 1984), and since DHT or GnRH-Ag treatment did not alter luteal estradiol production, changes in

the smooth endoplasmic reticulum may not be expected. The relationship between the shape of the inner mitochondrial membrane and the activity of $P\text{-}450_{scc}$ has not yet been clarified. It is not known if the availability of free cholesterol is essential for the integrity of the inner mitochondrial membrane or the latter can be affected without any changes in the former. We have two unique models in our laboratory where both treatments of DHT or GnRH-Ag lead to decreased luteal progesterone production, but only GnRH-Ag treatment selectively changes the morphology of the inner mitochondrial cristae. Therefore, it would be possible, by utilizing GnRH-Ag and DHT models, to determine the relationship between the shape of the mitochondrial membrane and the activity of its $P\text{-}450_{scc}$ as they relate to steroidogenesis in the corpus luteum.

In summary, the administration of DHT in pregnant rats results in acute high levels of DHT in the plasma, leading to decreased plasma progesterone levels which in turn inhibit the nocturnal surge of prolactin. The treatment may have caused the decreased luteal progesterone production by decreasing the processes that free cholesterol from cholesterol esters, or by decreasing the transport of cholesterol to mitochondria.

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Reconstitution of single calcium channels from secretory granules of rat adenohypophysis in planar lipid membranes

Erin Balden, Ameae M. Walker and Samuel Cukierman

Division of Biomedical Sciences, University of California, Riverside, CA 92521-0121, U.S.A.

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Key words: Ca^{2+} channel; Secretory granules; Adenohypophysis; Membrane, plasma, lipid; (Rat)

Summary

Free cytosolic Ca^{2+} ($[Ca^{2+}]_i$) has been demonstrated to play a crucial role in the prolactin secretory pathway. It regulates events as apparently diverse as prolactin gene transcription and the fusion of secretory granules with the plasma membrane. It is therefore important to understand the mechanisms which regulate the level of $[Ca^{2+}]_i$. Because prolactin secretory granule membranes have been shown to contain large amounts of Ca^{2+} and there is evidence that this calcium can be released independently of the granule hormone content, we have investigated the possibility that prolactin secretory granule membranes contain Ca^{2+} channels. When purified prolactin secretory granules were fused with an artificial phospholipid bilayer, we found a Ca^{2+} channel with a linear current–voltage relationship (conductance ~ 45 pS) in symmetrical 50 mM $CaCl_2$ solutions. Said channel had an open probability that was weakly dependent on the transmembrane potential, and a very good selectivity for calcium over chloride ions. The channel opened and closed very rapidly, when the majority of events lasting well below 25 ms. This channel could be important for the provision of high $[Ca^{2+}]_i$ levels necessary for granule-plasma membrane fusion and could also be involved in the modulation of Ca^{2+} fluxes across the plasma membrane after the exocytotic release of prolactin.

Introduction

Several important cellular processes are regulated by the concentration of free calcium in the cytoplasm ($[Ca^{2+}]_i$). One of those processes, essential for cell secretion, concerns the fusion of secretory vesicles with the plasma membrane. While the detailed molecular mechanisms by which $[Ca^{2+}]_i$ influences the fusion of secretory vesicles with the plasma membrane remain unknown, it has been demonstrated in nerve, as well as in different gland cells, that secretion of neurotransmitters and hormones follows an increase in $[Ca^{2+}]_i$ (Del Castillo and Katz, 1954; Schulz, 1980; Streb et al., 1983; Almers, 1990). In relation to pituitary cells, it has been additionally reported that $[Ca^{2+}]_i$ regulates the control of prolactin synthesis at the transcriptional level (Day and Maurer, 1990; Enyeart et al., 1990). In view of the central role played by calcium at all levels in the secretory pathway, it is therefore important to understand the different transport mechanisms involved in the control of $[Ca^{2+}]_i$ in pituitary cells.

Address for correspondence: Samuel Cukierman, Division of Biomedical Sciences, University of California, Riverside, CA 92521-0121, U.S.A.

Lorenson et al. (1990) found that the concentration of calcium in bovine pituitary secretory granules accounts for approximately 20% of total pituitary homogenate calcium. This amount of Ca^{2+} in secretory granules is in principle sufficient to account for variations known to occur in $[Ca^{2+}]_i$ during secretion although extracellular Ca^{2+} can be also considered a source during stimulus secretion coupling (Lewis et al., 1988; Almers, 1990). Lorenson et al. (1990) also observed Ca^{2+} fluxes across the granule membrane and suggested the presence of Ca^{2+} channels in the granule membrane. While their Ca flux study provided important information, the biochemical methods employed were limited by poor temporal resolution as well as by the lack of control over the potential across the granule membrane. These problems raise the issue of whether Ca^{2+} is really being transported by ion channels or by a different transport mechanism.

Ion channels from different intracellular organelles have been successfully reconstituted using the planar bilayer technique (see Miller, 1986). This method offers the possibility of studying single ion channels present in intracellular organelles that are too small to be studied with the patch clamp technique. The kinetics of single channel currents can be analyzed in the millisecond range while the membrane potential is controlled at the desired voltage. In this study, we have fused secretory granules from rat pituitary cells with planar bilayers. Using this technique, we provide the first description of the basic properties of Ca^{2+} channels present in the granule membrane. A possible physiological role for these granule channels is discussed.

Materials and methods

Subcellular fractionation of prolactin secretory granules

Pituitary subcellular fractionation was conducted as described by Zanini and Giannattasio (1973) with the following modifications: (1) the donor rats were female, Sprague-Dawley derived (Bantin and Kingman, Fremont, CA, U.S.A.), weighing approximately 250 g and at random stages of the estrous cycle; (2) the material present in the $15{,}000 \times g$ pellet, derived from 20 rats, was equally divided between two discontinuous sucrose gradients for further processing. Briefly, this protocol involves homogenization in 0.32 M sucrose, removal of debris and nuclei by a low-speed centrifugation, pelleting of organelles by a 30 min centrifugation at $15{,}000 \times g$, and treatment of the pellet with puromycin to: (a) detach ribosomes from rough microsomes and therefore increase their buoyancy, and (b) to produce lysis of most contaminating growth hormone granules. The puromycin-treated material is then loaded onto discontinuous sucrose gradients. This protocol produces a prolactin secretory granule fraction (fraction 3 on the discontinuous gradient) which is relatively free of contaminants, as indicated by ultrastructural and biochemical criteria (Zanini and Giannattasio, 1973; Greenan et al., 1989). The fraction was then washed by pelleting in 0.32 M sucrose containing 5 mM $MgCl_2$ and 1.0 M KCl at $30{,}000 \times g$ for 60 min and was finally resuspended in 0.5 ml of the supplemented sucrose solution. As described before, two-dimensional gel electrophoresis (see Fig. 1), and electron microscopic analyses of the subcellular fraction served as controls for purity (Greenan et al., 1989).

Artificial lipid membranes

Lipid bilayers were formed by painting a 0.15 mm diameter hole in a polystyrene partition separating two different compartments containing 2.5–3.0 ml. The lipid mixture was dissolved in purified decane (60 mg/ml) and had the following composition: 80% 1-palmitoyl-2-oleoyl-phosphatidylethanolamine and 20% 1-palmitoyl-2-oleoyl-phosphatidylcholine. These synthetic phospholipids were purchased from Avanti Lipids (Alabaster, AL, U.S.A.).

Solutions

Most experiments were carried out in 50 mM $CaCl_2$ symmetrical solutions (pH = 7.00, Hepes, 10 mM) at room temperature (21–23° C). In some experiments, the reversal potential of single ion channel currents was measured with a $CaCl_2$ gradient across the membrane. In this case, a control current voltage relationship was obtained in symmetrical $CaCl_2$ solutions after which one side of the membrane had the concentration of

$CaCl_2$ increased by adding a known amount of salt from a concentrated stock solution.

Electrophysiology

One side of the membrane was kept at ground potential (*cis*-side) while the other side (*trans*-side) was voltage-clamped. The membrane potential in this study was defined as ($V_{trans} - V_{cis}$). This would be analogous to the potential difference between the intragranular and cytoplasmic sides across the granule membrane. Both solutions were connected to the amplifier by means of 3 M KCl-agar bridges. Junction potentials were measured and found to be less than 1–2 mV. Ionic currents were measured with a List patch-clamp amplifier (List Electronics, Darmstadt, F.R.G.). Single channel currents were digitized at 1 kHz using a Labmaster AD-board (Scientific Solutions, Solon, OH, U.S.A.). Commercially available software (p-Clamp, Axon Instruments, Sunnyvale, CA, U.S.A.) was employed for analyzing single channel recordings. The open probability of the channel was calculated by the mean open and closed times and/or by the average current method (for a more detailed description, see Cukierman, 1990; Cukierman and Krueger, 1990).

Experimental procedure

Once a bilayer was formed between the two different compartments, intact secretory granules (core plus membrane, ~ 50 μl of the final suspension) were added only to the *cis*-side of the membrane. The membrane was voltage clamped at 50 mV and the *cis*-side was stirred for variable lengths of time. The incorporation of ion channels in the bilayer was detected by a sudden jump in the bilayer conductance and by monitoring the opening and closing events normally associated with channel function.

Results

Fig. 1 shows a silver-stained two-dimensional gel of the proteins present in the whole cell homogenate (*A*) and in the secretory granule fraction (*B*). Prolactin is the major protein present. Both monomer and oligomer prolactin are evident. Proteins 'a' and 'b' are added molecular weight and p*I* markers corresponding to carbonic anhydrase (p*I* 5.9 and molecular weight 29 kDa) and β-lactoglobulin-A (p*I* 5.1 and molecular weight 18.4 kDa), respectively. A much smaller amount of growth hormone is present in the gran-

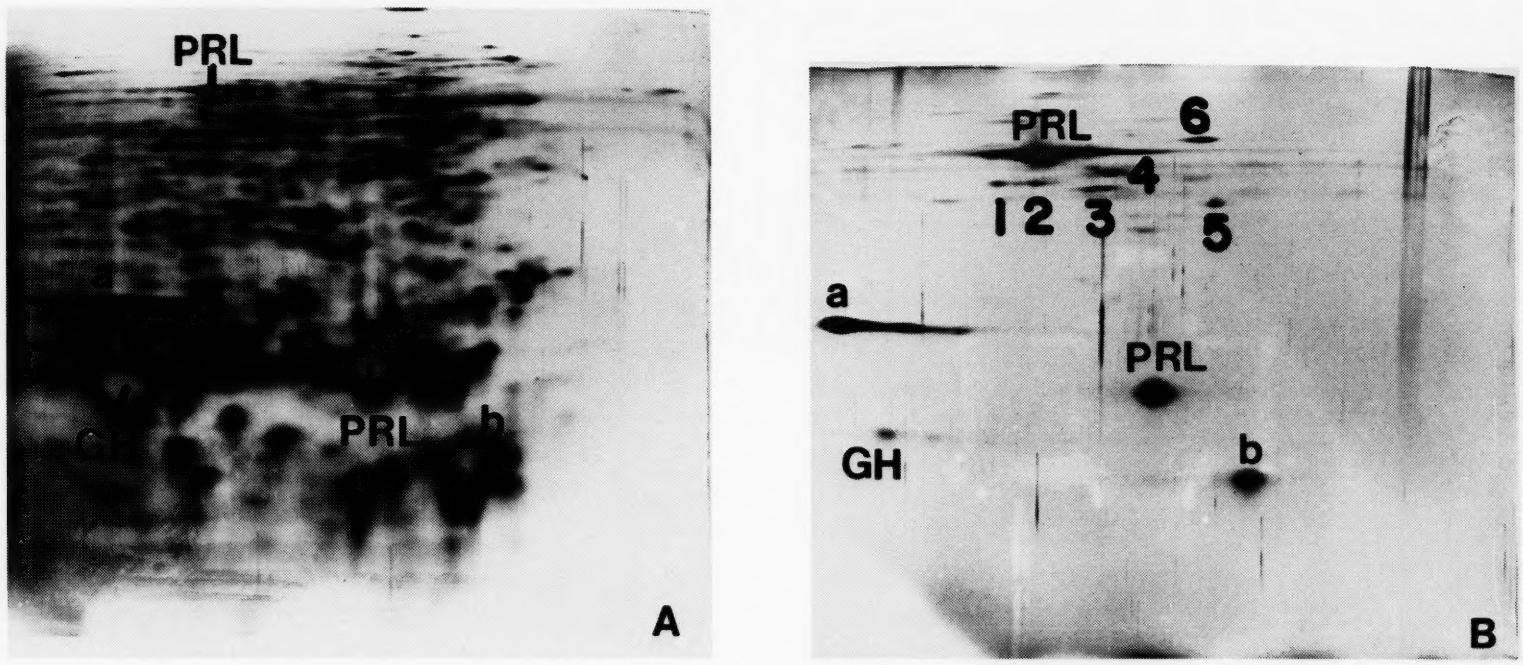

Fig. 1. Silver-stained two-dimensional gels of the proteins present in a pituitary homogenate (*A*) and in the secretory granule fraction (*B*). PRL, prolactin; GH, growth hormone; a, b, molecular weight and p*I* markers; proteins 1–6, granule associated proteins as explained in the text.

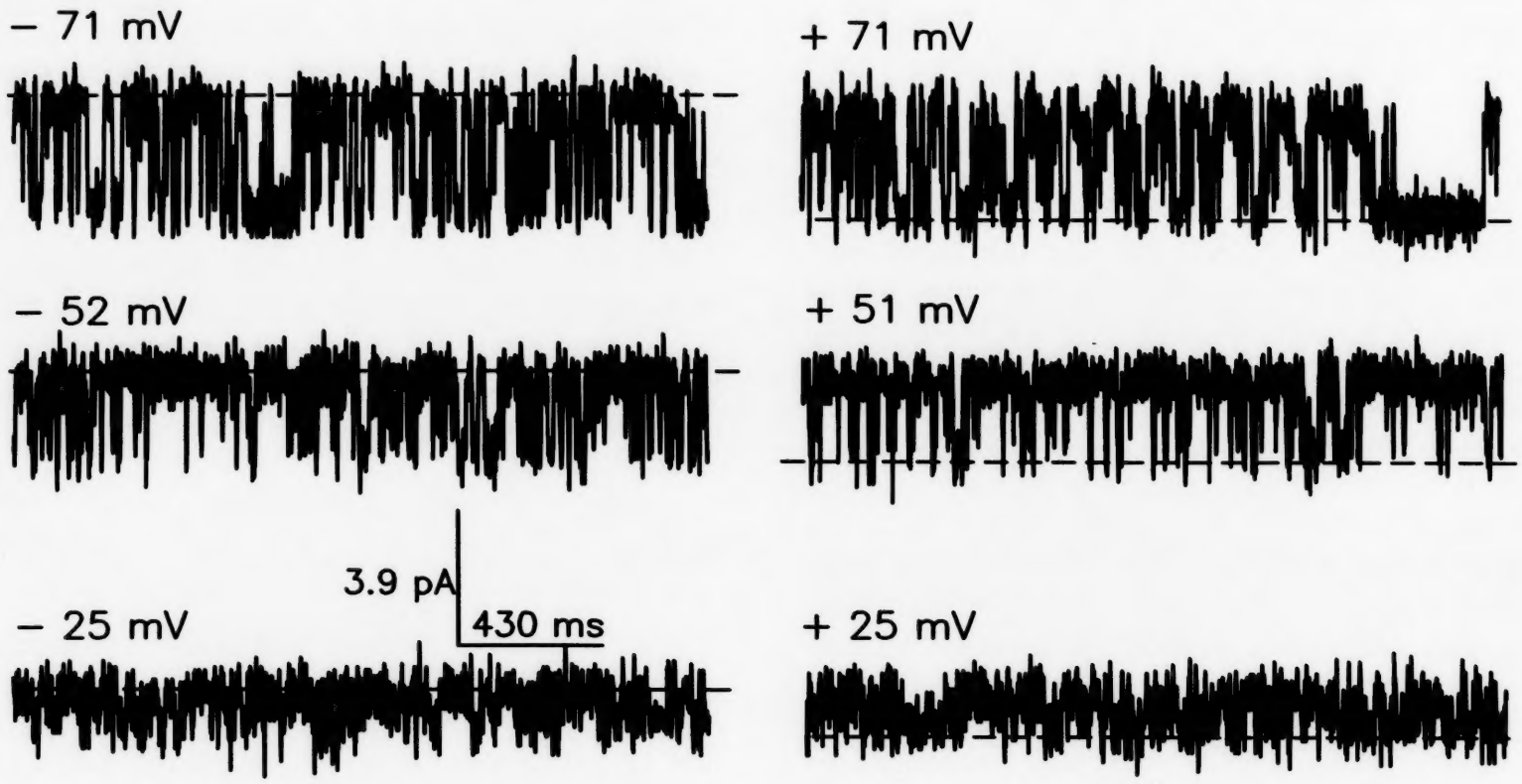

Fig. 2. Single channel recordings at different transmembrane voltages (indicated at the upper left corner of each recording). The closed state of the channel is indicated by a dashed line in each recording and corresponds to the zero current level. Current recordings were obtained in symmetrical 50 mM $CaCl_2$ solutions.

ule fraction versus the whole cell homogenate. Thus the fractionation procedure markedly favors the isolation of prolactin over growth hormone granules, despite their very similar size and density. Only seven of between 150–200 substantial spots present in the whole cell homogenate are left in the granule fraction in addition to the prolactin and growth hormone spots. Six of these spots are present in the same proportion to prolactin as they are in the whole cell homogenate (cf. 1–6 in *B*), and they are lost when the granule membrane is removed (data not shown). It seems therefore that they are granule membrane or peripheral granule core proteins. Given the sensitivity of the silver staining procedure and the ability of the two-dimensional gel system to discriminate among hundreds of different proteins, these results demonstrate a very pure preparation of secretory granules.

Fig. 2 shows sample recordings of a single Ca^{2+} channel in a bilayer at different membrane potentials. The dashed line in each recording represents the closed state of the channel (zero current level). It can be seen that the channel opens and closes in the millisecond time scale with rare long (> 50 ms, see also Fig. 5) duration openings or closures at different membrane potentials.

Fig. 3 shows single channel current–voltage relationships. Circles represent single ion channel

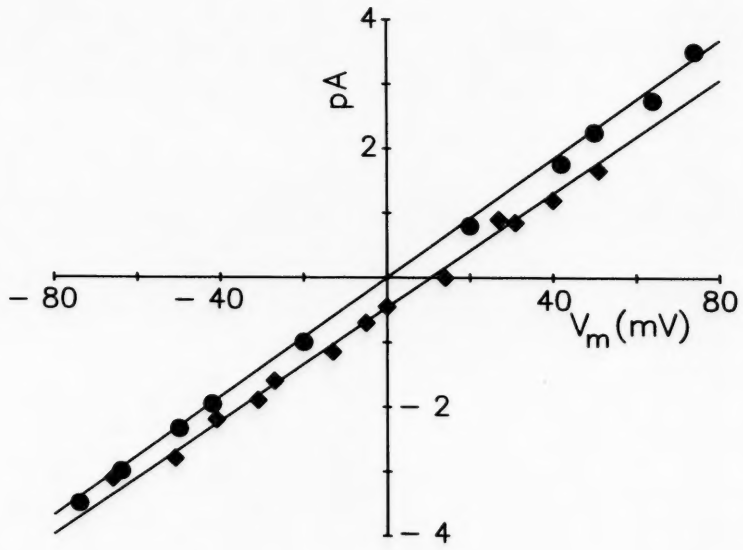

Fig. 3. Single channel current–voltage relationship in symmetrical 50 mM $CaCl_2$ solutions (circles) and after increasing the $CaCl_2$ concentration on the *cis*-side to 140 mM (squares). Lines were drawn following regression analyses and the reversal potential in asymmetrical salt solutions was found to be +9 mV.

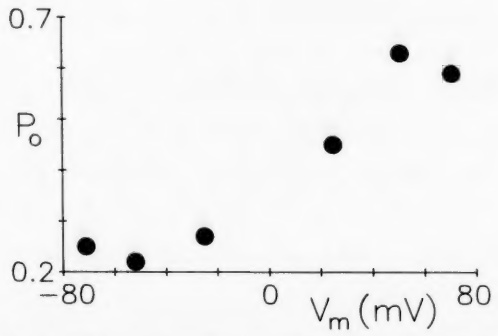

Fig. 4. Relationship between membrane potential (V_m) and the open probability of the channel (P_0).

currents in symmetrical 50 mM $CaCl_2$ solutions across the membrane while squares represent currents after the *cis*-side of the channel had its $CaCl_2$ concentration increased from 50 to 140 mM. In the experimental conditions tested, the channel transports calcium ions in both directions with the same efficiency: the channel has an ohmic behavior with a conductance of ~45 pS. The Nernst relation predicts an electrochemical potential of 10 mV for such a 140/50 mM Ca^{2+} gradient across the membrane. The regression line fitting the experimental points (squares) crosses the abscissa at 9 mV. This indicates that the channel has a very good selectivity for Ca^{2+} over Cl^- ions.

In the range of membrane potentials tested, the voltage dependence of the channel's open probability (P_0) was not found to be 0 (completely closed at all times) or 1 (completely open at all times). Channel openings were found to be weakly dependent on transmembrane voltage. These points are illustrated in Fig. 4. In this figure, the

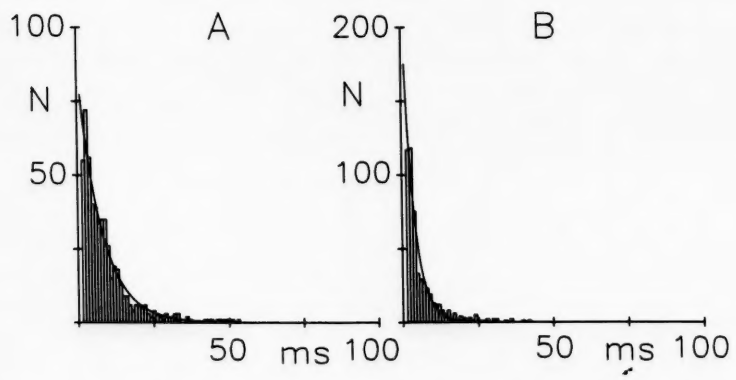

Fig. 5. Dwell time histograms of the open (*A*) and closed (*B*) states of the channel. Data are from open and closed distributions of a single Ca^{2+} channel. The membrane potential was 75 mV. Both dwell time distributions are fitted by single exponential curves (time constants: 7.63 ms in *A*, mean open time; 3.38 ms in *B*, mean closed time).

channel open probability is plotted against the transmembrane potential. As suggested by the recordings of Fig. 2, the open probability of the channel increased from ~0.27 at −75 mV to ~0.7 at 70 mV.

Fig. 5 shows dwell time distributions of open (*A*) and closed (*B*) events of the Ca^{2+} channels. Both open and closed dwell times were fitted by single exponentials. This suggests that a minimum kinetic scheme compatible with the gating of the channels comprises a single open and closed state.

Discussion

The first question that we would like to address is: What is the direct experimental evidence favoring the view that the Ca^{2+} channels presented in this study really originate from the granule membrane? Although one can produce extremely pure subcellular fractions, it is impossible to eliminate all contaminating membranes. When studying single reconstituted ion channels, one can therefore not be absolutely sure that they derived from the major component of the subcellular fraction. However, several aspects of this study, combined with previous results of others, suggest that the channels in question did indeed arise from the secretory granule membrane. First, the degree of purification of the granule preparation was high and therefore, given the normal fusogenic properties of the secretory granules, it is likely that the channels derived from granule membranes. Second, in a recent study, Enyeart et al. (1990) studied the basic electrophysiological characteristics of Ca^{2+} currents in the plasma membrane of GH cells and Lewis et al. (1988) studied them from normal anterior pituitary cells. These currents were found to be extremely sensitive to the membrane potential and to show a significant inactivation. These characteristics are not shared by our single channel recordings. Third, in some experiments with the plasma membrane fraction of pituitary cells, we did not detect the presence of the described channels in our bilayers. Unfortunately, there is no direct way to address this question experimentally. If it were possible to patch clamp these submicroscopic granules, then there would be no doubt. Also, if the fusion of a single secretory granule contributed a measurable average

aliquot of prolactin to the *trans*-side of the bilayer then one could also correlate this to the presence of channels. Given the current methods available for measuring prolactin however, this, like patch clamping, is an impractical approach. Thus, we have investigated the properties of Ca^{2+} channels in the granule membrane by the most direct (at the single channel level) and feasible method at present. We have found a Ca^{2+} channel that displays an ohmic behavior, a weak dependence of the open probability on the transmembrane potential and a very good selectivity for calcium over chloride ions.

What could be the physiological function of Ca^{2+} channels in the granule membrane? Since it has been reported that the content of intragranular Ca^{2+} is relatively high (Lorenson et al., 1990), one possibility is that those channels could be chemically activated in the secretory process leading to a local increase in $[Ca^{2+}]_i$ that is important for fusion of granules with the plasma membrane. Another interesting possibility that we would like to consider concerns the regulation of prolactin synthesis and secretion by $[Ca^{2+}]_i$. It has been shown that $[Ca^{2+}]_i$ is able to modulate the synthesis in addition to the release of prolactin by pituitary cells (Day and Maurer, 1990; Enyeart et al., 1990). Thus, it is attractive to think that after a massive fusion of granules with the plasma membrane leading to release of prolactin from the cells, the plasma membrane would have an increase in Ca^{2+} channel density contributed by the granule membranes. Since the described channels are weakly voltage dependent, their presence in the plasma membrane could lead to an increased calcium permeability that would in turn raise $[Ca^{2+}]_i$ and trigger the synthesis of prolactin. Such a mechanism would constitute an interesting feedback loop by which synthesis of prolactin would follow prolactin depletion in the cell. Further studies to confirm the importance of these channels to the physiology of pituitary cells must await the discovery and/or design of probes that selectively affect the functioning of those channels.

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Type 1 and 2 isoenzymes of cAMP-dependent protein kinase in Leydig cell steroidogenesis

R. William Hipkin and William H. Moger

Departments of Physiology and Biophysics, and Obstetrics and Gynecology, Dalhousie University, Halifax, Nova Scotia B3H 4H7, Canada

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Summary

This study examined the functional significance of the type 1 (T1) and type 2 (T2) cAMP-dependent protein kinase (PK-A) isoenzymes in androgen production by mouse Leydig cells. Leydig cells were exposed to cAMP analogues selective for either of the two cAMP binding sites on the regulatory subunits of each PK-A isoenzyme. As the two binding sites have been shown to exhibit positive cooperativity, coexposure to the appropriate combination of analogues will synergistically increase androgen production if either T1 or T2 PK-A is present and functional in the cell. We found that both PK-A isoenzymes are present and functionally active, though the T1 kinase predominates. Coexposure to the cAMP analogues and cAMP or luteinizing hormone also synergistically increased androgen production via both isoenzymes while forskolin acted only via the T1 isoenzyme, suggesting that forskolin may instigate cellular events in addition to cAMP synthesis.

Introduction

It has been well documented that luteinizing hormone (LH) from the anterior pituitary controls steroidogenic activity in the Leydig cell through the cyclic adenosine 3′,5′-monophosphate (cAMP) second messenger system (Mendelson et al., 1975; Cooke et al., 1976). However, gonadotropin-stimulated androgen production has been detected prior to any discernible elevation in intracellular cAMP levels in normal (Mendelson et al., 1975) and tumour Leydig cells (Peirera et al., 1987). The ability of the inactive cAMP analogue, R_p-cAMP, to inhibit the steroidogenic response to levels of LH which do not initiate any discernible increase in cyclic nucleotide levels does, however, suggest that the cAMP cascade is involved (Peirera et al., 1987). The study by Peirera et al. (1987) further suggested that the type 1 (T1) isoenzyme of cAMP-dependent protein kinase (PK-A) was functionally dominant in the mouse tumour Leydig cells though the type 2 (T2) enzyme could be isolated. The purpose of our study was to characterize the functional role of the two major isoenzymes of PK-A in the control of steroidogenic mechanisms in the normal murine Leydig cell. To do so, we have utilized analogues of cAMP which are selective for either of the two cAMP binding

Address for correspondence: William H. Moger, Department of Physiology and Biophysics, Dalhousie University, Halifax, Nova Scotia B3H 4H7, Canada.

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sites (S1 and S2) on the regulatory subunits of the T1 or T2 PK-A isoenzymes. Each PK-A isoenzyme is a heterotetramer comprised of a homodimer of regulatory subunits associated with two catalytic subunits which are released and activated upon cAMP binding to the regulatory dimer. The two nucleotide binding sites on the regulatory proteins have been shown to exhibit positive cooperativity (Corbin et al., 1988), in that binding at one site increases binding at the other site. Therefore, coexposure of the mouse Leydig cell to aminohexylamino-cAMP (AHA), an S1/T1-selective analogue and N^6-benzoyl-cAMP (B), an analogue selective for S2/T1 or T2 should elicit a synergistic increase in androgen production if type 1 PK-A is present and functional in the cell. Alternatively, the presence of the T2 isoenzyme would become evident should a nonadditive steroidogenic response result upon combined exposure to an S1/T2 analogue, 8-thiomethyl-cAMP (TM), and the site 2 type nonselective analogue (B). Using these cAMP analogues in combination or coincubated with LH, cAMP or forskolin, we have ascertained that both PK-A isoenzymes are present and functionally important in murine Leydig cell steroidogenesis.

Materials and methods

The AHA, B and TM cAMP analogues, cAMP, forskolin, TC-199, $NaHCO_3$, *N*-(2-hydroxyethyl) piperazine-*N'*-(2-ethanesulfonic acid) (Hepes), L-glutamine, pyruvate and bovine serum albumin (BSA) were purchased from Sigma Chemicals (St. Louis, MO, U.S.A.). Collagenase derived from *Clostridium hystolyticum* was purchased from Boehringer Mannheim (Laval, PQ, Canada). NIH-LH-B9 (0.7 × NIH-LH-S1) was a generous gift from Dr. L. Reichert, Jr. and the NIAMDD Pituitary Hormone Distribution Program.

Cell isolation and purification

Mouse Leydig cell preparations were prepared as previously described (Murphy and Moger, 1982). Briefly, adult male mice (Charles River, 25–30 g) were sacrificed, castrated and the decapsulated testes dispersed enzymatically with gentle shaking for approximately 15 min at 34° C (0.33 mg type 1 collagenase and 10 mg bovine serum albumin (BSA; fraction V) per millilitre of tissue culture medium 199 (TC-199)). The TC-199 was further augmented to contain 27 mM sodium bicarbonate, 10 mM Hepes, 0.7 mM L-glutamine and 1 mM pyruvate.

Enzymatic dispersion was terminated by the addition of cold (4° C) TC-199 and the tubular elements were removed by filtration of the testicular homogenate through nylon mesh (44 and 72 μm). The interstitial cell suspension was then centrifuged (120 × *g* for 5 min), resuspended in TC-199, layered on continuous Percoll gradients (Pharmacia, Dorval, PQ, Canada) and centrifuged for 15 min at 900 × *g*. The Leydig cell layer was removed with a 35 ml syringe, diluted with media (1 : 3) and centrifuged (120 × *g*). The cell pellet was washed 3 times, resuspended in 2 ml of TC-199 and the Leydig cell number assessed using a haemocytometer.

Leydig cell incubations

Leydig cells (20,000 cells/500 μl final volume of TC-199) were incubated in 12 × 75 mm polystyrene, round-bottomed tubes for 3 h. A warm (34° C), oxygenated (95% O_2, 5% CO_2) and humidified atmosphere was maintained with a shaking water bath (80 oscillations/min). Chemical mediators were dissolved in saline or TC-199 such that the desired treatment levels could be attained with additions of 10 μl/tube. The incubations were terminated by centrifugation (1000 × *g* for 5 min) and the medium was stored at −20° C.

Radioimmunoassay

Testosterone production was measured by radioimmunoassay in unextracted samples using an antiserum generously provided by Dr. D.T. Armstrong (University of Western Ontario) as described by Anakwe and Moger (1986). Due to crossreactivity with other 17β-hydroxy-androgens, all results refer to total androgen production.

Synergism ratio

As the nucleotide binding sites on the regulatory subunits exhibit positive cooperativity, activation of a PK-A isoenzyme by pairs of selective analogues is revealed through a synergistic rather than additive increase in androgen produc-

tion. This synergistic increase in steroidogenesis is expressed as a synergism ratio (SR) such that SR = (XY)–C/(X–C) + (Y–C) where XY is the steroidogenic response upon coexposure to a combination of two agents X and Y, and C represents the basal androgen production in the absence of any stimulatory agent. Therefore, SR > 1 is indicative of a synergistic response while a SR = 1 represents an additive response.

Statistics

The synergism ratio data were assessed for statistical significance using a single mean Student's *t*-test with $p \leq 0.05$ considered to be statistically significant.

Results

The effect of type / site selective analogues of cAMP on androgen production

In experiment 1, Leydig cells were incubated with AHA (1 μM; S1, T1), TM (10 μM; S1, T2) or B (10 μM; S2, T1/2) alone or in combinations to selectively activate type 1 PK-A (AHA + B), type 2 PK-A (TM + B) or both isoenzymes (AHA + TM). All of these treatment paradigms stimulated androgen production with the analogue combinations acting in a synergistic manner to increase steroidogenic output (Table 1, Fig. 1). Calculation of synergism ratios (as described in Materials and Methods) reveals that activation of either isoenzyme of PK-A resulted in a significant synergistic increase in androgen production (PK-A type 1 SR = 5.62 ± 0.72 SEM, $p \leq 0.05$; type 2 SR = 4.26 ± 0.77, $p \leq 0.05$). Coexposure to analogues which bind to S1 of each isoenzyme (AHA + TM) should not elicit a synergistic response, though a significant degree of interaction is evident (SR = 2.65 ± 0.35, $p \leq 0.05$). This finding illustrates the difference between selectivity and specificity of the cAMP analogues. The structural alterations within the cyclic nucleotide derivatives increase their type/site selectivity above that of endogenous cAMP without providing absolute specificity for a particular binding site or isoenzyme.

TABLE 1

THE EFFECT OF SITE/TYPE SELECTIVE cAMP ANALOGUES, ALONE AND IN COMBINATION, ON ANDROGEN PRODUCTION BY MOUSE LEYDIG CELLS

Treatment	Androgen (ng/million cells/3 h) [a]
Control	69.23 ± 13.3
1 μM AHA cAMP	150.3 ± 30.6
10 μM B cAMP	138.7 ± 26.7
10 μM TM cAMP	125.6 ± 25.2
AHA + B	820.2 ± 137.5
B + TM	509.5 ± 90.0
AHA + TM	477.8 ± 44.8

[a] Mean ± SEM of six separate experiments.

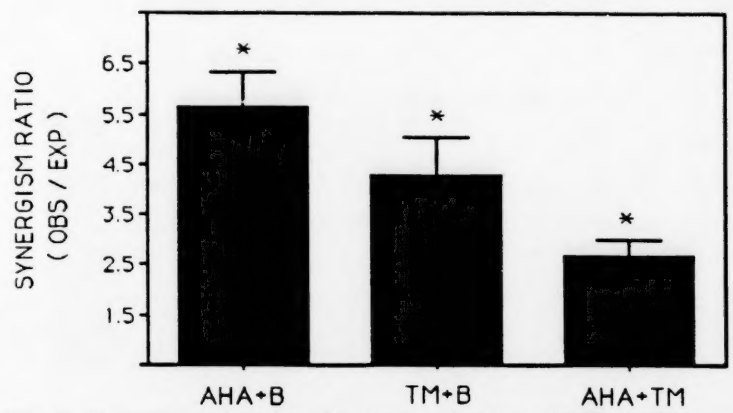

Fig. 1. Synergism between site/type selective cAMP analogues. Increase in androgen production upon coexposure of mouse Leydig cells to AHA (S1, T1) + B (S2, T1/2), TM (S1, T2) + B and AHA + TM. Note: *y*-axis in this figure is the synergism ratio (SR) calculated as described in Materials and Methods. * Significant statistical difference from additive response (SR = 1.0), $p \leq 0.05$. Data represents the mean ± SEM of six separate experiments (five determinations/experiment).

The effect of phosphodiesterase inhibition on the steroidogenic response to the site / type selective cAMP analogues

In experiment 2, we addressed the possibility that the site/type selective cAMP analogues could be enhancing steroidogenesis through inhibition of cyclic nucleotide phosphodiesterase (PDE) activity. With this in mind, the steroidogenic effects of the analogues were assessed in the presence or absence of 0.1 mM 1-isobutyl-3-methylxanthine (MIX), a PDE inhibitor. If the steroidogenic response to the cAMP analogues was mediated through sparing of cyclic nucleotides then chronic PDE inhibition should reduce the response. As illustrated in Fig. 2, PDE inhibition with MIX had no effect on the response to the cAMP analogues,

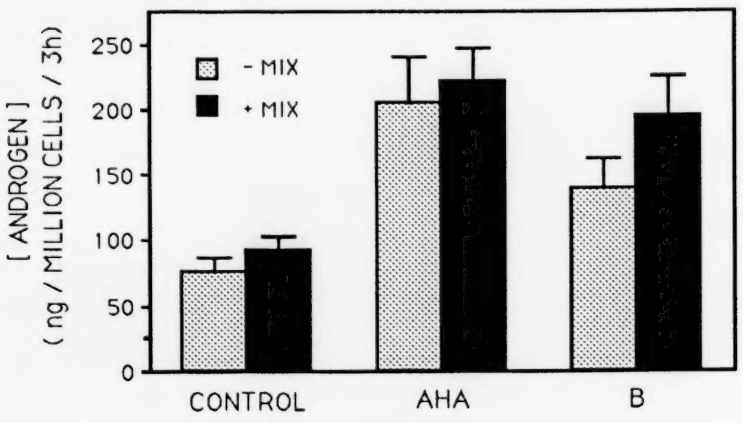

Fig. 2. The effect of phosphodiesterase inhibition on the steroidogenic response to AHA and B. Increase in androgen production upon exposure of mouse Leydig cells to AHA (1 μM) or B (10 μM) $\pm$ 0.1 mM MIX. Data represents the mean $\pm$ SEM of four separate experiments.

suggesting that the sparing of cyclic nucleotides was not a factor in the response.

Interaction between cAMP and the site / type selective analogues

In experiment 3, each of the cAMP analogues was combined with exogenous cAMP (1 mM) or endogenous cAMP generated through activation of adenylate cyclase with the diterpene forskolin (0.5 μM). Both of these treatment paradigms were capable of eliciting a minimal cAMP-mediated increase in androgen production (control 58.5 $\pm$ 11.6 vs. forskolin 74.55 $\pm$ 12.19; control 63.28 $\pm$ 10.4 vs. cAMP 77.1 $\pm$ 12.5 ng androgen/million cells/3 h). As can be seen in Fig. 3, a synergistic response was evident with the cells coexposed to exogenous cAMP, predominantly through the PK-A 1 isoenzyme though significant interaction via the type 2 isoenzyme was evident (AHA SR = 2.56 $\pm$ 0.65, $p \leq 0.04$; B SR = 1.75 $\pm$ 0.22, $p \leq 0.012$; TM SR = 1.54 $\pm$ 0.24, $p \leq 0.05$). The addition of 0.5 μM forskolin also elicited a synergistic increase in androgen production (Fig. 3) albeit somewhat smaller than that noted with cAMP and predominantly through the type 1 PK-A holoenzyme (AHA SR = 1.57 $\pm$ 0.2, $p \leq 0.025$; B SR = 1.50 $\pm$ 0.24, $p \leq 0.05$; TM SR = 1.12 $\pm$ 0.13, $p \leq 0.19$).

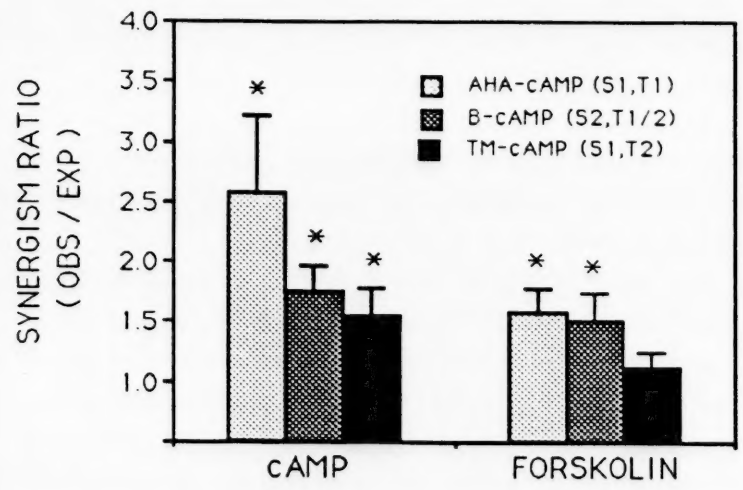

Fig. 3. Synergism between site/type selective cAMP analogues and exogenous cAMP or forskolin. Synergistic increase in androgen production upon coexposure of mouse Leydig cells to AHA (S1, T1), B (S2, T1/2) or TM (S1, T2) and 1.0 mM cAMP or 0.5 μM forskolin. * Significant statistical difference from additive response (SR = 1.0), $p \leq 0.05$. Data represents the mean $\pm$ SEM of four separate experiments (five determinations/experiment).

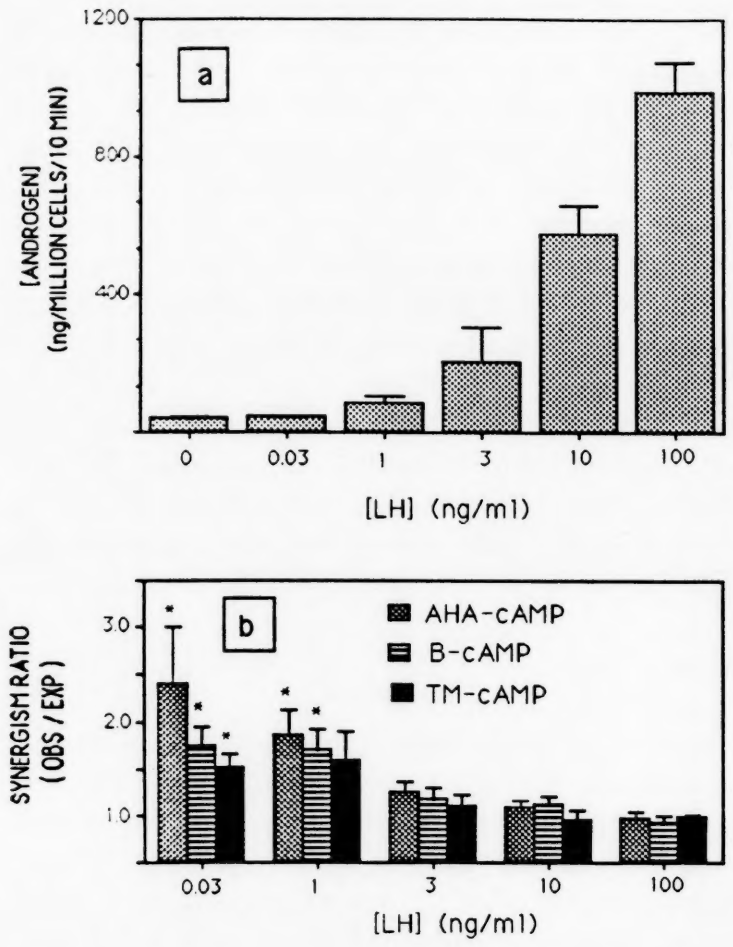

Fig. 4. Synergism between site/type selective cAMP analogues and LH. (*a*) Androgen production in response to LH (0.03–100 ng/ml). (*b*) Synergistic increase in androgen production upon coexposure of mouse Leydig cells to AHA (S1, T1), B (S2, T1/2) or TM (S1, T2) and LH. * Significant statistical difference from additive response (SR = 1.0), $p \leq 0.05$. Data represents the mean $\pm$ SEM of four separate experiments (five determinations/experiment).

Interaction between LH and site / type selective analogues

In experiment 4, the Leydig cells were treated with LH (0.3–100 ng/ml) alone, or in combination with each of the site/type selective cAMP analogues (Fig. 4*a* and *b*). In this case, a significant synergistic interaction is evident with low levels of LH via both protein kinase isoenzymes (Fig. 3), with interaction through type 1 PK-A predominating (0.3 ng/ml LH: AHA SR = 2.40 ± 0.6, $p \leq 0.05$; B SR = 1.75 ± 0.2, $p \leq 0.02$; TM SR = 1.51 ± 0.15, $p \leq 0.025$; 1.0 ng/ml LH: AHA SR = 1.86 ± 0.27, $p \leq 0.025$; B SR = 1.70 ± 0.22, $p \leq 0.025$; TM SR = 1.58 ± 0.31, $p \leq 0.73$).

Discussion

It is well established that LH is the predominant stimulatory mediator of Leydig cell steroidogenic function and that this control is manifested through the cAMP second messenger system (Mendelson et al., 1975; Cooke et al., 1976; Dufau et al., 1987). The development of cAMP analogues selective for the binding sites on the regulatory subunits of two isoenzymes of cAMP-dependent protein kinase has allowed for a more thorough examination of the next step in the cAMP cascade. Peirera and coworkers (1987) were able to identify both type 1 and 2 PK-A from the cytosol of cultured murine Leydig tumour cells but found that type 2 PK-A was responsible for only a minor percentage of the detectable phosphotransferase activity (15–20%). Our findings suggest, however, that the type 2 isoenzyme does have a significant physiological role in the mouse Leydig cell exceeding that which might be construed from the PK-A type 2 phosphotransferase activity measured in the mouse tumour cell. Coexposure to analogues selective for the two nucleotide binding sites on the type 2 regulatory units (TM and B) consistently resulted in significant synergistic increase in androgen production albeit somewhat reduced from that resulting from type 1 activation (AHA and B; Table 1, Fig. 1). This would indicate that both PK-A isoenzymes are present and functionally active in the mouse Leydig cell. The steroidogenic effect of the cAMP analogues was not mediated by PDE inhibition as inclusion of the PDE inhibitor, MIX, had no effect on the response (Fig. 2).

A physiological role for both PK-A isoenzymes is supported by the synergistic interaction via both type 1 and 2 PK-A noted upon coexposure of the cells with exogenous cAMP (Fig. 3) or low stimulatory levels of LH (Fig. 3). The lack of synergism at higher but submaximal concentrations of LH is not surprising as the cAMP analogues are competing with endogenously generated cyclic nucleotides for the binding sites on the regulatory subunits of the kinase isoenzymes. As the synthesis of cAMP increases with increased LH stimulation of adenylate cyclase (Mendelson et al., 1975; Cooke et al., 1976), the selective effect of the analogues is lessened as the competition for binding sites escalates (Corbin et al., 1988). By this same reasoning, to elicit the maximal degree of synergism, the site/type selective cAMP analogues should be utilized at concentrations which consistently but minimally stimulate androgen production (Table 1).

It is interesting to note that the magnitude of interaction between cAMP and the PK-A isoenzymes seemingly varies with the means by which the cyclic nucleotide is introduced into the cell. Synergistic interaction with the analogues was much more prevalent with cAMP provided exogenously (Fig. 3) or generated endogenously with LH (Fig. 4) than that achieved upon coexposure to forskolin (Fig. 3), particularily through the type 2 isoenzyme. The lack of synergism between forskolin and the type 2 selective analogue, relative to that noted with LH/cAMP, is difficult to explain as all three treatment paradigms seemingly stimulate steroidogenesis via the same cAMP messenger cascade. However, the possibility exists that forskolin/adenylate cyclase interaction (Seamon et al., 1981) could reflect only one of the functional activities of the diterpene. Recently, it has become apparent that forskolin has a number of functions independent of adenylate cyclase activation (Laurenza et al., 1989) including inhibition of glucose transport (Joost et al., 1988) and modulation of ion channels (Coombs and Thompson, 1987; Wagoner and Pollatta, 1988). Forskolin may invoke changes in the intracellular environment which are not optimum for cooperative activation of the cAMP-dependent kinases in general, and

the type 2 holoenzyme in particular. This could explain the inability of forskolin to maximally stimulate androgen production to the same extent as LH in the mouse Leydig cell (Moger and Anakwe, 1983). More recent studies noted that although cAMP generation and binding to the regulatory subunit were comparable, exposure to 1 μM forskolin was less effective than human chorionic gonadotropin (hCG) in stimulating steroidogenesis (Dufau et al., 1987). Indeed, lower (pM) levels of forskolin actually inhibited both cAMP generation and androgen production in the rat Leydig cell (Dufau et al., 1987) while higher concentrations (≥ 3.12 μM) inhibited the transport of 2-deoxy-D-glucose (Amrolia et al., 1988). Further investigations of cAMP-independent activities of forskolin in the murine Leydig cell are certainly warranted.

It is apparent that the two isoenzymes of cAMP-dependent protein kinase are present and active in the murine Leydig cell and that both the type 1 and 2 isoenzymes play a part in the stimulatory control of androgen production by LH. It is also apparent that forskolin may have cAMP-independent activities which reduce the ability of the cyclic nucleotide to synergistically activate steroidogenesis through both PK-A isoenzymes.

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High density lipoprotein interaction with human placenta: biochemical and ultrastructural characterization of binding to microvillous receptor and lack of internalization

Eliane Alsat [1] and André Malassiné [2]

[1] *INSERM U.166, Endocrinologie de la Reproduction, Maternité Baudelocque, 75014 Paris, France, and Laboratoire de Physiopathologie du Développement CNRS, Ecole Normale Supérieure, 75230 Paris, France, and* [2] *Laboratoire de Physiologie Cellulaire, URA 290, Service Universitaire de Microscopie Electronique, UFR Sciences, 86022 Poitiers Cedex, France*

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Summary

Specific receptor and internalization process for low density lipoprotein (LDL) and modified LDL (acetyl-LDL) have been well characterized in placental microvilli and in trophoblastic cells in culture. The aim of this study was to investigate high density lipoprotein (HDL_3) binding and its eventual subsequent internalization in both these purified placental preparations. Isolated term placental microvilli were used for binding of [^{125}I]HDL_3 (devoid of apoprotein E). HDL_3 were conjugated to colloidal gold for ultrastructural visualization of binding and internalization in syncytiotrophoblast in culture. Saturable binding of HDL_3 was identified. Scatchard analysis revealed a K_d value of 24.2 ± 8.0 μg HDL_3 protein/ml and a maximum binding capacity at 4°C of 128.2 ± 54.5 μg HDL_3 protein/mg of membrane protein. These sites have broad specificity: both LDL and acetyl-LDL were able to partially inhibit the HDL_3 binding. Ultrastructural study confirms that gold-HDL_3 bind specifically to syncytiotrophoblast membrane. However, after incubation at 37°C, an internalization process similar to those described for gold-LDL and gold-acetyl-LDL was not observed for gold-HDL_3. These results demonstrate specific HDL_3 binding without internalization. The physiological significance of an HDL_3 membranous interaction and the placental steroidogenesis remains to be established.

Introduction

The human placenta possesses a high rate of steroid and membrane biosynthesis, which requires an important supply of maternal cholesterol, derived from circulating lipoproteins (Simpson and Burkhart, 1980; Gwynne and Strauss, 1982). Specific high affinity receptors for low density lipoprotein (LDL) and for biochemically modified LDL have been characterized in human placenta (Cummings et al., 1982) and more precisely on the microvillous membranes (Alsat et al., 1982, 1985; Malassiné et al., 1984) which represent the effective site of exchange between the maternal blood and the placental tissue. LDL and acetyl-LDL receptors are two distinct binding proteins (Rebourcet et al., 1986) with molecular masses of 180 kDa and 200 kDa, respectively. They were de-

Address for correspondence: Dr. A. Malassiné, Laboratoire de Physiologie Cellulaire, URA 290, UFR Sciences, 86022 Poitiers Cedex, France.

tected on placental microvilli, as early as the 6th week of pregnancy (Alsat et al., 1984, 1987). Ultrastructural visualization of the LDL and acetyl-LDL internalization by trophoblast cells in culture has been reported (Malassiné et al., 1987, 1990). In addition to LDL binding, saturable VLDL binding to human placental membranes has been described (Naoum et al., 1987).

Interestingly, high density lipoprotein (HDL) may also participate in the cholesterol transport, the first step of this process being an interaction of HDL particles with a cell membrane receptor. HDL subclasses vary in their apoprotein composition and degree of heterogeneity (Einserberg, 1984) and these particles exist in a continual state of flux as a result of their complex role in the overall metabolism of lipoproteins. Binding and metabolism studies need the use of HDL_3 subfraction, devoid of apolipoprotein E (Apo E) to prevent any interaction with the LDL receptor (Apo B/E receptor). Binding sites for HDL_3 particles were firstly identified in a mixed placental plasma membrane prepared from human placenta (Cummings et al., 1982), in purified microvillous membrane (Alsat et al., 1988) and more recently in isolated trophoblast cells (Jorgensen et al., 1989). Evidence that human placenta possesses a receptor protein for HDL has been provided by ligand blotting analysis of crude placental membrane preparations, using HDL_3 (Graham and Oram, 1987) or Apo A-1 (Keso et al., 1987) as ligand. However, the mechanism of uptake and possible metabolism of HDL by human placenta is very poorly understood. Further investigations including morphologically and physiologically well-defined trophoblastic components are necessary for fine receptor characterization and fine localization. Indeed, the human hemomonochorial placenta is characterized by complex anatomical and biochemical interactions between fetal and maternal tissues. Thus, the main functional structure, the chorionic villi, consists of trophoblasts surrounding a mesenchymal core of connective tissue including fibroblasts, Hofbauer cells (macrophages), endothelial and smooth vascular cells. The trophoblast itself comprises an incomplete layer of mononucleated cytotrophoblasts and an external uninterrupted layer of multinucleated syncytiotrophoblasts. The microvillous membrane of the syncytiotrophoblast is in direct contact with maternal blood.

We have, therefore, attempted to characterize and visualize the interaction of HDL_3 with two different purified placental preparations. Isolated placental microvilli were used to characterize the $[^{125}I]HDL_3$ binding, and cultured syncytiotrophoblast for ultrastructural localization of binding sites and potential subsequent internalization process of gold-conjugated HDL_3 particles. Results strongly suggest that HDL_3 binds to high affinity receptor sites located on the microvillous membrane of syncytiotrophoblast without being internalized into cellular compartments. A part of this study has been previously reported (Alsat et al., 1988, 1989).

Material and methods

Placental microvilli preparation

Term placentae were collected immediately after elective ceasarean section or vaginal delivery

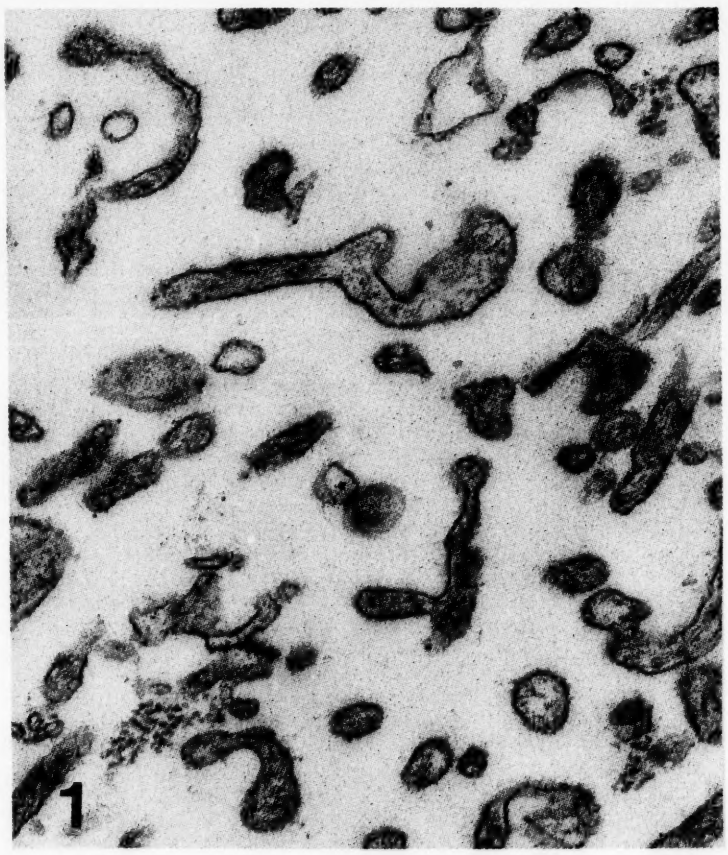

Fig. 1. Electron micrograph of purified microvillous fraction prepared from normal term placenta. The microvilli contain electron-dense material with longitudinally oriented filaments (×26,000).

from healthy mothers. The placental microvilli were isolated by cold saline extraction and centrifugations as detailed elsewhere (Alsat et al., 1982) and stored at −80 °C until use. Our previous reports have assessed the purity of the membrane preparations by enzyme marker analysis and by electron microscopy examinations: compared to whole placental homogenate, the isolated microvillous fractions where found to be 14- to 17-fold enriched in 5′-nucleotidase, and 17- to 25-fold in alkaline phosphatase, a specific marker for placental microvilli. The ultrastructural study indicated that these preparations were predominantly constituted by placental microvilli (Fig. 1).

Trophoblast cell culture

Cytotrophoblasts were isolated from human term placentae obtained immediately after uncomplicated ceasarean section using a method previously described (Malassiné et al., 1987) similar to that of Kliman et al. (1986). Briefly, villous tissue was dissected free of membranes and vessels, rinsed and minced in calcium and magnesium-free Earle's balanced salt solution. The minced tissue was subjected to several trypsin-DNase digestions at 37 °C in Ca^{2+}-free Earle's solution containing 0.25% trypsin (Trypsin 1 : 250 Difco), 100 Kunitz units/ml DNase, 25 mM Hepes and 4.2 mM $MgSO_4$ pH 7.4. The resultant cell suspensions were submitted to a Percoll gradient (5–70%) centrifugation step. After centrifugation at 1200 × *g* for 20 min the middle layer of cells was removed, washed once with Ham's F10 medium and then diluted to a concentration of 1×10^6 cells/ml in Ham's F10 supplemented with 15% heat-inactivated fetal calf serum and antibiotics: 100 μg/ml streptomycin and 100 U/ml penicillin. The culture medium was renewed every 24 h. Twenty-four hours before binding and internalization experiments, the culture medium was replaced by a medium without fetal calf serum.

Cell culture was characterized by scanning electron microscopy (Fig. 2) immunohistochemistry of αhCG, βhCG, cytokeratin and by βhCG and progesterone secretion (Malassiné et al., 1990).

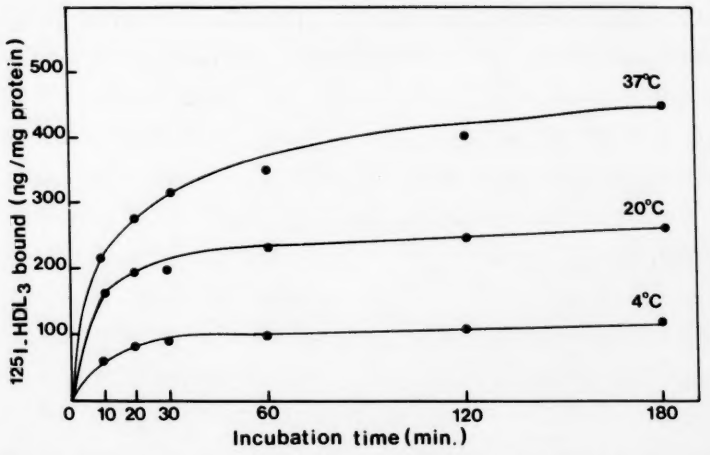

Fig. 3. Time course of specific [^{125}I]HDL_3 binding to human placental microvilli, at different incubation temperatures. Membrane aliquots (60 μg protein) were incubated for the indicated incubation time at 4 °C, 20 °C or 37 °C with 14.75 μg/ml [^{125}I]HDL_3 (450 cpm/ng) in the absence (total binding) or presence of 1 mg/ml unlabelled HDL_3 (non-specific binding). Each point represents the mean value of specific binding calculated by subtracting non-specific from total binding determined in triplicates.

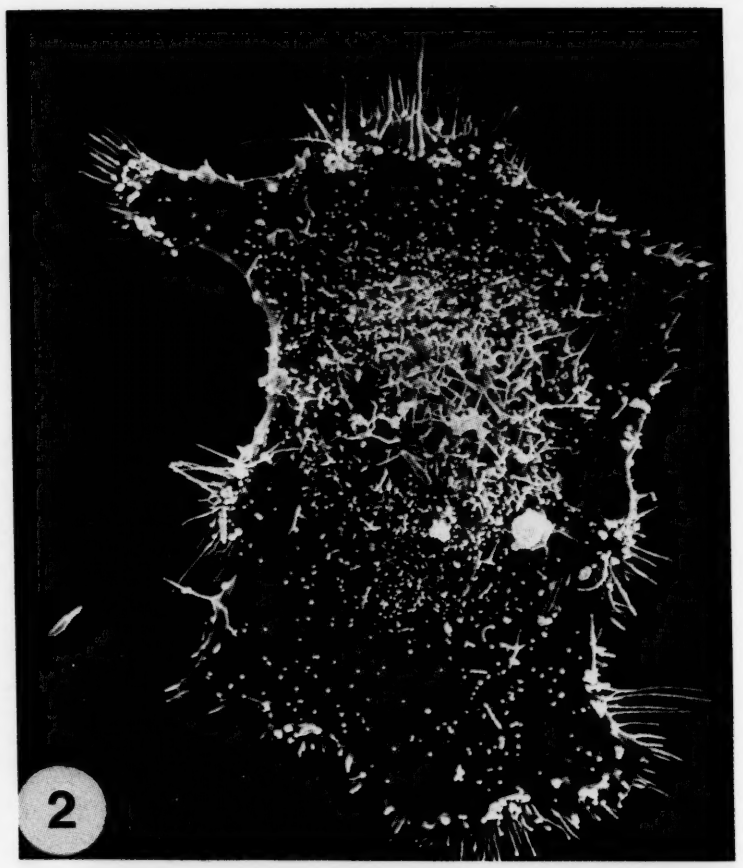

Fig. 2. Scanning electron micrograph of syncytiotrophoblast obtained 3 days after the plating of cytotrophoblastic cells. Note the central nuclear mound, the extending cytoplasm, the cytoplasmic extensions and the microvilli (×1400).

Lipoproteins: isolation, iodination and gold conjugation

Human LDL (*d* 1.024–1.050 g/ml) and HDL_3 (*d* 1.125–1.215 g/ml) were isolated from freshly

obtained normolipidaemic plasma of non-pregnant donors by sequential ultracentrifugation (Havel et al., 1955). Acetyl-LDL were prepared by treatment of native LDL by acetic anhydride, as previously described (Alsat et al., 1985). The lipoprotein fractions were dialysed against a solution containing 5 mM Tris, 50 mM NaCl and 0.04% EDTA (pH 7.4). The absence of Apo E contamination of HDL_3 subclass was assessed by sodium dodecyl sulphate polyacrylamide gel electrophoresis. This was further assessed by the complete lack of HDL_3 competitive effect with [^{125}I]LDL for the binding to placental microvilli, whereas 100 μg/ml of total HDL fraction (d 1.070–1.215 g/ml) inhibited the [^{125}I]LDL binding by 40%. HDL_3 were iodinated using the iodine monochloride method (Bilheimer et al., 1972) to a specific activity of 475–530 cpm/ng.

Colloidal gold with a mean particle diameter of 15 nm was prepared by the method of Frens (1973). Conjugation of HDL_3 to the negatively charged gold particles was performed following the method of Handley et al. (1981) modified by Robeneck et al. (1982). To 5 ml of colloidal gold, 0.5 ml of freshly dialysed HDL_3 (100 μg protein HDL_3) was added and mixed rapidly. The conjugate was separated by centrifugation at 9000 × g for 1 h against 40% sucrose, dialysed overnight against an isotonic buffer and used within 24 h. The complex stability was tested by incubation with 0.5 M NaCl solution.

[^{125}I]HDL_3 binding assays

The binding of [^{125}I]HDL_3 to isolated placental microvillous membranes was determined by a method similar to that previously used for the characterization of [^{125}I]LDL and [^{125}I]acetyl-LDL binding sites in placental microvilli (Alsat et al., 1982, 1985). Specific binding was calculated as the difference between total and non-specific bindings measured in the absence or presence of an excess of unlabelled HDL_3 ($\simeq$ 0.8 mg/ml), respectively. All binding determinations were carried out in triplicate and the reported experiments were per-

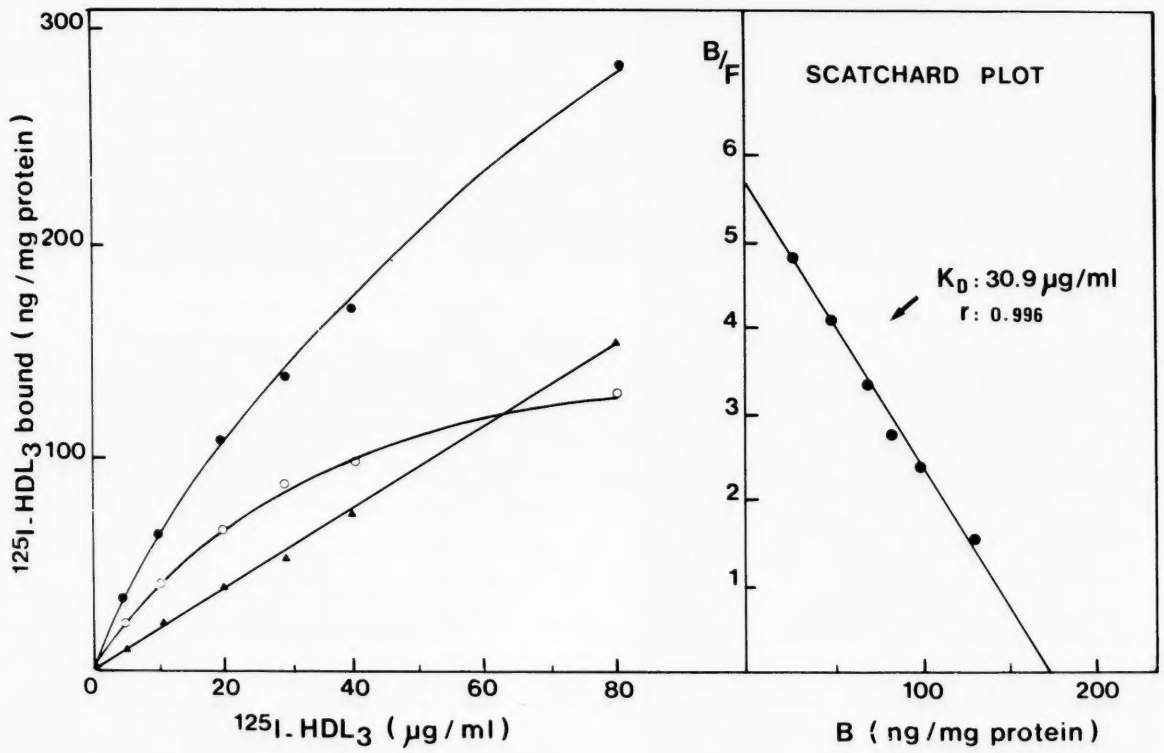

Fig. 4. Saturation curve of [^{125}I]HDL_3 binding to human placental microvilli and Scatchard plot of this [^{125}I]HDL_3 specific binding. Membrane aliquots (67 μg protein) were incubated for 1 h at 4°C with increasing amounts of [^{125}I]HDL_3 (500 cpm/ng) in the absence (total binding) or presence (non-specific binding) of 0.864 mg/ml unlabelled HDL_3. Each point is the mean of triplicate determinations and specific binding was calculated by subtracting non-specific binding from total binding. The apparent K_d was 30.9 μg protein HDL_3/ml and the maximal binding capacity (B_{max}) was 176.1 ng protein [^{125}I]HDL_3/mg of microvillous protein.

formed at least 3 times. Protein content was determined according to the method of Lowry et al. (1951) using bovine serum albumin as a standard.

Incubation procedure with gold-HDL_3 and electron microscopy

Cells were prechilled, washed 3 times with Ham's F10 medium and incubated as previously described (Malassiné et al., 1990). Briefly, for binding experiments the cells were incubated at 4°C for 2 h in a medium containing gold-HDL_3 conjugate (40–60 μg HDL_3 protein/ml). Control cells were incubated with gold-HDL_3 in the presence of an excess of unlabelled HDL_3 (400 μg/ml medium). For internalization experiments the cells were continuously incubated with gold-HDL_3 in medium at 37°C for 5, 15, 60 min for the running experiments. In the pulse-chase procedure the cells were allowed to bind gold-HDL_3 for 2 h at 4°C, washed twice with phosphate-buffered saline, and then warmed to 37°C in Ham's F10 medium, to internalize the membrane-bound lipoprotein complex. For electron microscopy the cells were fixed 30 min with 2.5% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.4) and post-fixed with 1% OsO_4 in cacodylate for 30 min at 4°C. The cells were then dehydrated in a graded ethanol series and embedded in araldite. Ultrathin sections were stained with uranyl acetate and lead citrate. The lengths of the plasma membrane components and the distribution of gold-HDL_3 particles were quantified on micrographs by a Kontron Interactive Image Analysis System as previously described (Malassiné et al., 1984; Mommaas-Kienhuis et al., 1985).

Results

Placental microvilli and syncytiotrophoblast characterization

Fig. 1 shows a microvillous-enriched fraction. The microvilli contain electron-dense material with longitudinally oriented filaments. When isolated cytotrophoblasts were cultured in 15% supplemented fetal calf serum medium, after 2 or 3 days multinucleated cells identical to syncytiotrophoblast were observed (Fig. 2). The cell surface exhibited numerous microvilli, coated and non-coated pits and cytoplasmic protrusions (Figs. 6, 7, and 8). The cytoplasm contains the usual cytoplasmic organelles and a well-developed endocytic apparatus: coated and non-coated vesicles, endosomes, multivesicular bodies and lysosome-like dense bodies.

[^{125}I]HDL_3 binding to microvillous membranes

The time course study of specific [^{125}I]HDL_3 binding was examined at 4, 20 and 37°C (Fig. 3). Equilibrium was reached between 60 and 80 min, and remained constant for up to 180 min. This indicates the presence of temperature-dependent saturable binding sites for HDL_3. In these experiments the non-specific binding is unaffected by temperature.

Fig. 4 illustrates a representative binding experiment of increasing amounts of [^{125}I]HDL_3 to placental microvillous membranes in the presence and absence of an excess of unlabelled HDL_3. The specific binding of [^{125}I]HDL_3 is a saturable process with an half-maximum binding occurring at a concentration of approximately 30 μg/ml. The same pattern of saturation curve was observed for

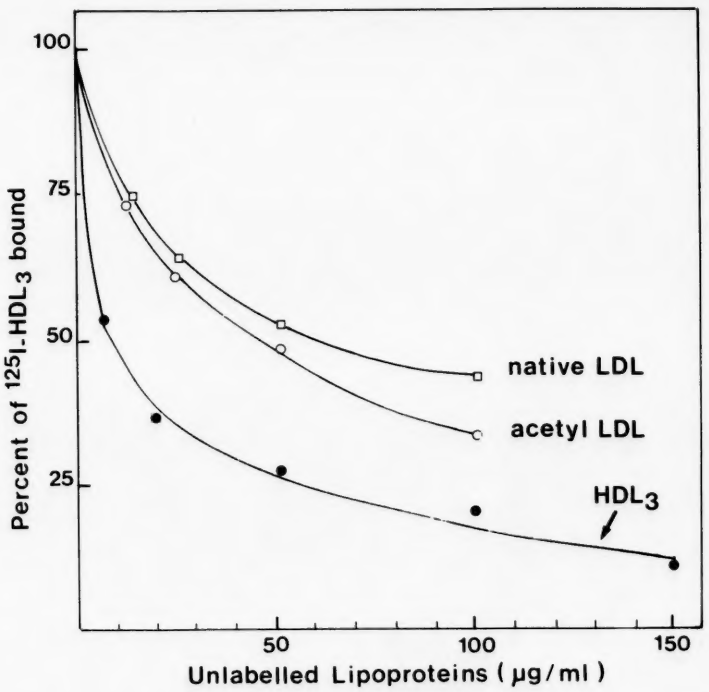

Fig. 5. Effect of different unlabelled lipoproteins on the [^{125}I]HDL_3 binding to human placental microvilli. Membrane aliquots (30 μg protein) were incubated for 1 h at 4°C with 7.4 μg/ml [^{125}I]HDL_3 (450 cpm/ng) and increasing concentrations of unlabelled HDL_3 (●), native LDL (□) or acetyl-LDL (○). Each value is the mean of triplicate determinations and is corrected for non-specific binding determined in triplicate parallel assays in the presence of 1 mg/ml unlabelled HDL_3.

TABLE 1

BINDING OF GOLD-HDL_3 TO 500 μm PLASMA MEMBRANE OF HUMAN SYNCYTIOTROPHOBLAST IN CULTURE

The number of gold-HDL_3 binding sites was assessed by random measurement on electron micrographs from four binding and internalization experiments. At least 500 μm of membrane of each experimental procedure was measured, and bound gold-HDL_3 particles were counted if localized within 80 nm from the plasma membrane.

Incubation procedures	Number of bound particles or aggregates			
	Microvilli	Intermicro-villous space	Coated pits	Total number
Binding experiment				
Gold-HDL_3 (60 μg/ml) 4°C for 2 h	121	59	12	192
Gold-HDL_3 (60 μg/ml) + HDL_3 (400 μg/ml) 4°C for 2 h	7	5	2	14
Running experiment				
Gold-HDL_3 (60 μg/ml) 37°C for 60 min	144	48	10	202
Pulse-chase experiment				
Gold-HDL_3 (60 μg/ml) 4°C for 2 h; 37°C for 60 min	187	26	5	218

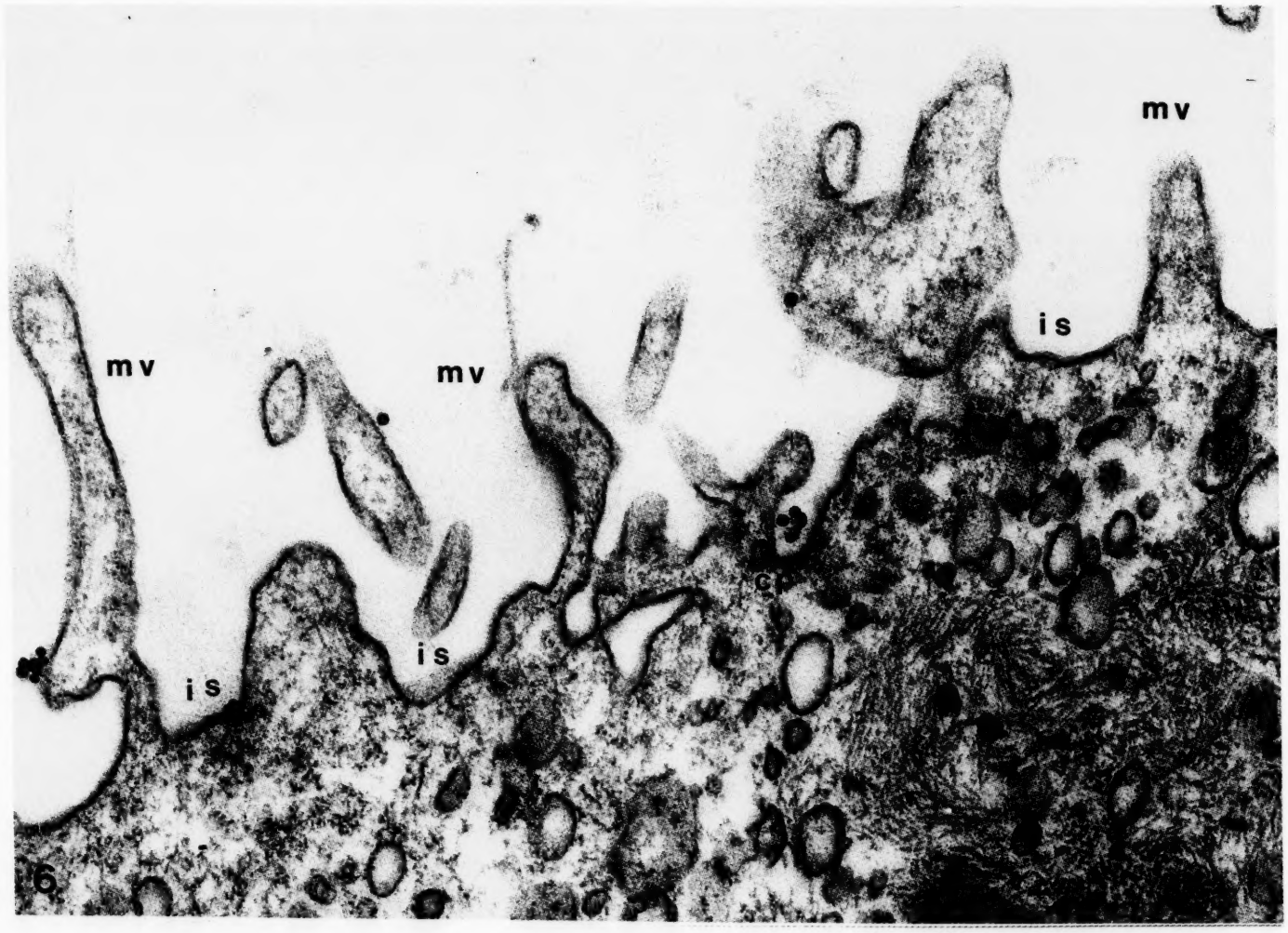

Fig. 6. Syncytiotrophoblast incubated for 2 h at 4°C with gold-HDL_3 (60 μg protein/ml of medium) and then washed twice with phosphate-buffered saline. The particles of gold conjugates are sparsely scattered over the surface of microvilli (mv), the intervillous space (is) and coated pits (cp) (×46,500).

higher concentrations of HDL_3 up to 120 μg protein/ml. Scatchard analysis of this saturation curve (Fig. 4) reveals an apparent single class of binding sites with a K_d value of 30.9 μg HDL_3 protein/ml and a maximal binding capacity (B_{max}) of 176.1 ng HDL_3 protein/mg of membrane protein. Using different batches of [^{125}I]HDL_3 with microvillous membranes from seven different placentas, the Scatchard analysis of the specific binding data indicates an apparent K_D value of 24.2 $\pm$ 8.0 μg HDL_3 protein/ml (mean $\pm$ SD, n = 7) and a B_{max} value at 4°C of 128 $\pm$ 54.5 ng/mg HDL_3 protein/ml of membrane protein (mean $\pm$ SD, n = 7).

To determine whether this binding was specific for HDL_3, the effect of LDL and acetyl-LDL was examined. Fig. 5 shows that in the presence of increasing concentrations of lipoprotein, both LDL and acetyl-LDL at equimolar concentrations are able to inhibit the binding of iodinated HDL_3 to placental microvilli. However, LDL and acetyl-LDL inhibited the HDL_3 binding significantly but incompletely.

Gold-HDL_3 binding to syncytiotrophoblast in culture

After 3 days of culture and 24 h in fetal calf serum-free medium, the syncytiotrophoblast incubated for 2 h at 4°C with HDL_3-conjugates showed gold-HDL_3 complexes attached to the plasma membrane. The particles of gold-HDL_3 were observed either as unitary or aggregate forms. These particles were sparsely scattered over the surface of microvillous, intermicrovillous spaces and over the membrane surface of the 'coated pits' (Table 1 and Fig. 6). When labelled HDL_3 was coincubated with an excess of unlabelled HDL_3 the binding of gold-HDL_3 was rarely seen: 14 particles on 500 μm of membrane compared with 192 particles on 500 μm of membrane incubated with gold-HDL_3 alone (Table 1 and Fig. 7).

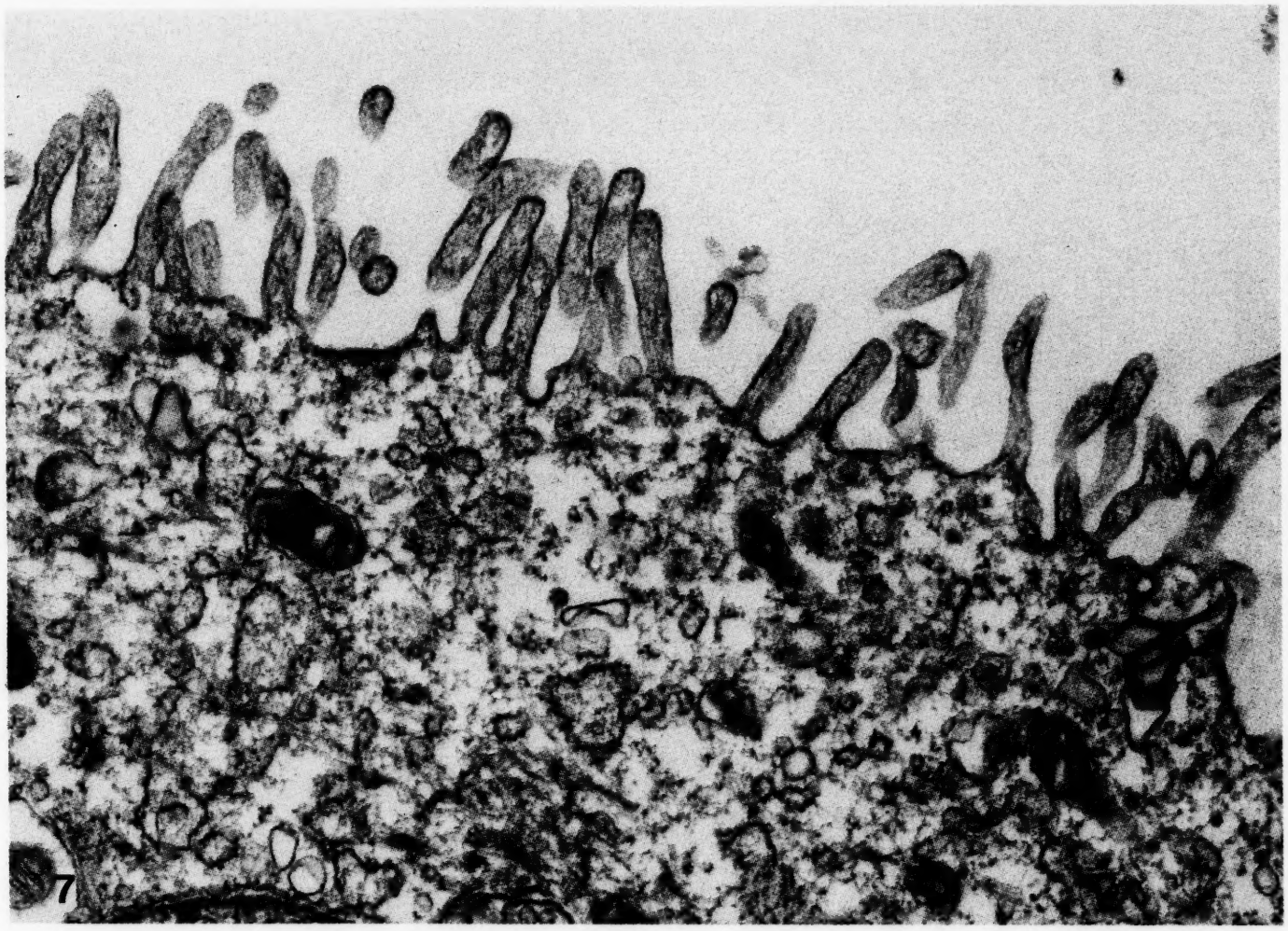

Fig. 7. Syncytiotrophoblast incubated for 2 h at 4°C with gold-HDL_3 (60 μg protein/ml of medium) in the presence of an excess of unlabelled HDL_3 (400 μg/ml of medium). Gold-HDL_3 binding is not observed (×27,000).

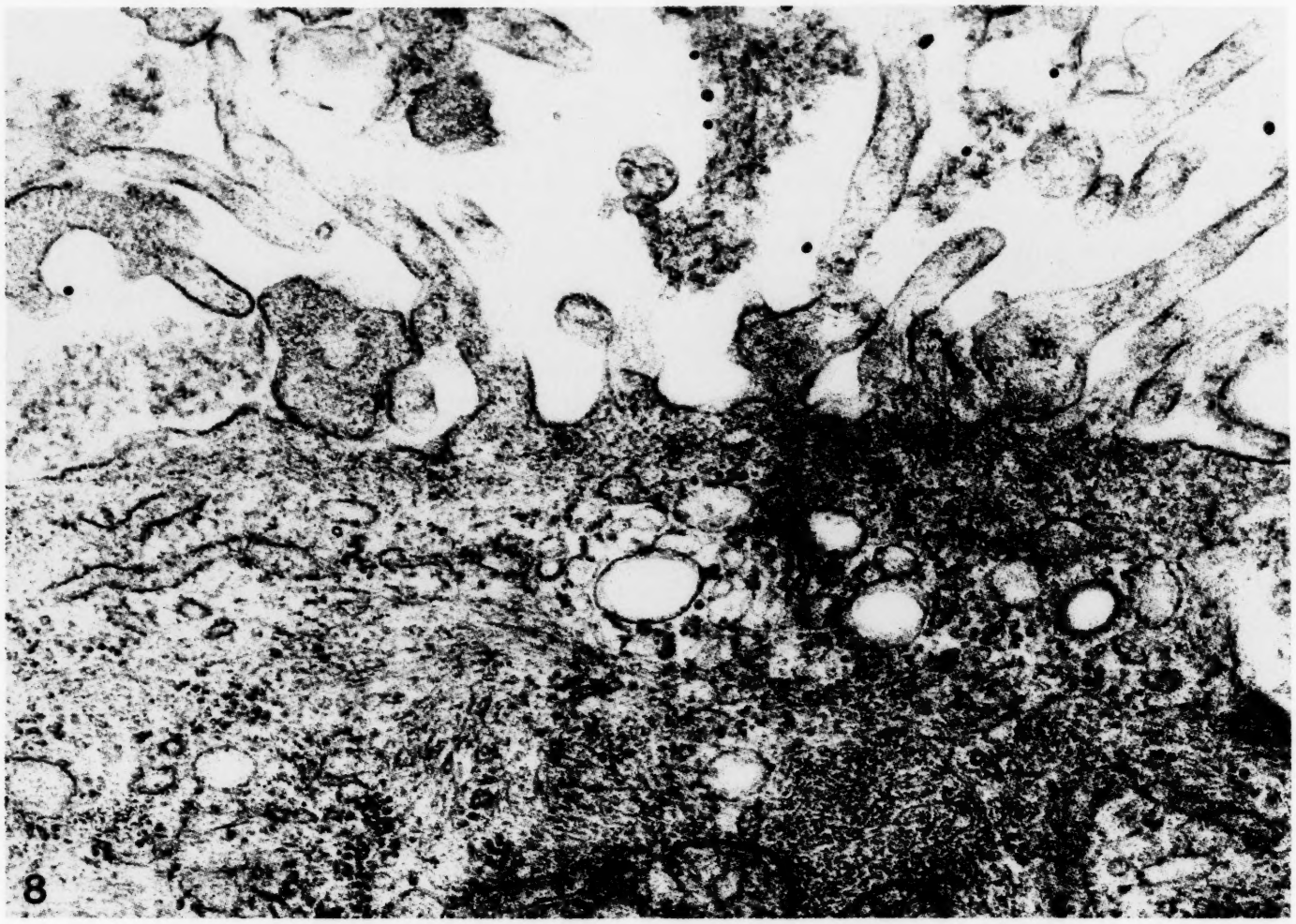

Fig. 8. Syncytiotrophoblast incubated continuously with gold-HDL_3 (60 μg protein/ml of medium) at 37° C for 60 min. Gold-HDL_3 particles are still located on the plasma membrane (×43,000).

To visualize a potential route for gold-HDL_3 internalization, running and pulse-chase experiments were performed, at 37° C. After an incubation of 60 min at 37° C, gold-HDL_3 particles were still located on the plasma membrane with random distribution (Table 1). No endocytosis was observed in cultured syncytiotrophoblasts, either after the running experiment or after the pulse-chase experiment (Fig. 8).

However, in an experiment with unpurified placental cells (Percoll gradient omitted) macrophages exhibited gold-HDL_3 particles endocytosis in their endosomal compartment (Fig. 9), showing the biological efficiency of the HDL_3 conjugates.

Discussion

Our results clearly demonstrate: (i) the presence of HDL_3 receptors on highly purified placental microvillous membranes and on syncytiotrophoblast in culture; (ii) the lack of subsequent endocytosis of receptor-gold-HDL_3 conjugates.

The binding affinity constant reported here is similar to the value obtained for high affinity binding ($K_d = 9.7$ μg/ml) to isolated trophoblast cells in suspension (Jorgensen et al., 1989). It is different from those reported for placental membranes (Cummings et al., 1982) and for isolated microvilli (Naoum et al., 1987). However, the placental membranes used by Cummings et al. (1982) were prepared from crude placental homogenate containing membranes from all placental components (trophoblast, fibroblasts, macrophages, endothelial cells, smooth vascular cells and blood cells). For their study Naoum et al. (1987) used a highly purified microvillous membrane preparation with a HDL fraction ranging in density from 1.00 to 1.40 g/ml. This fraction contains Apo E which bound with high affinity to LDL receptor (Apo B/E receptor). As described

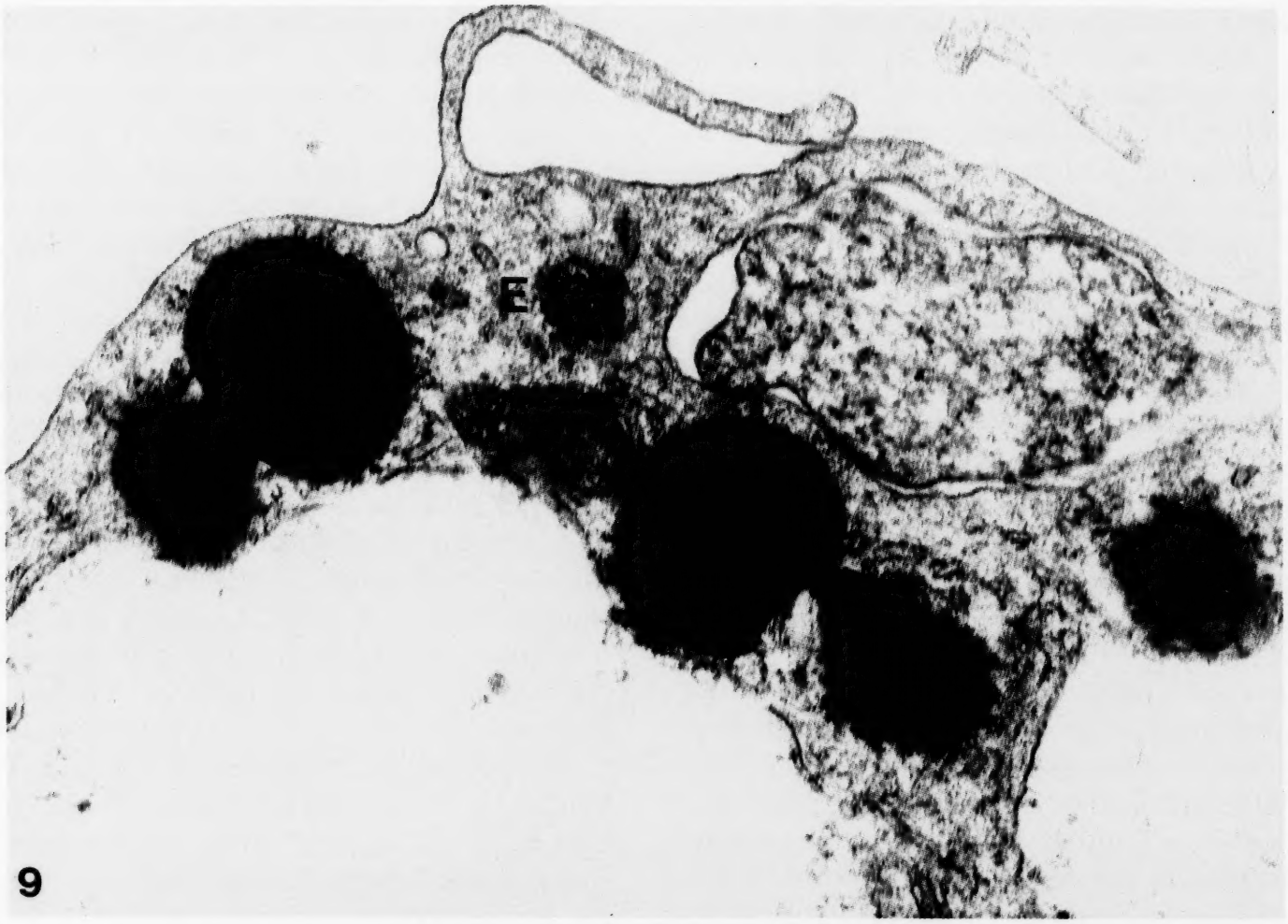

Fig. 9. Placental macrophage (from unpurified placental cell culture) incubated continuously with gold-HDL_3 (60 μg protein/ml of medium) at 37°C for 10 min. Gold-HDL_3 particles are located in endosome (E) ($\times$40,000).

for other cell membranes and trophoblast cells (Jorgensen et al., 1989), HDL_3 binding to purified microvillous membranes is temperature sensitive.

The binding sites have broad specificity; a similar result has been reported for the competitive effect of LDL on HDL_3 binding to trophoblast cells in suspension (Jorgensen et al., 1989). These findings suggest that in human syncytiotrophoblast LDL and HDL particles bound to multiple recognition sites or to different conformations of the same lipoprotein binding domain of the membrane. HDL binding sites lacking specific apoprotein recognition have been described in numerous human cells: skin fibroblasts (Biesbroeck et al., 1983), endothelial cells (Havekes et al., 1984) and adipocytes (Fong et al., 1985). To identify the HDL determinant(s) which interact with cell membrane sites, Tabas and Tall (1984) studied the ability of synthetic phospholipid-cholesterol vesicles containing either Apo A-1 or Apo A-II or no protein, to compete with labelled HDL_3 for binding to three different culture cells. They conclude that association of HDL_3 is not mediated by specific ligand and receptor proteins, but involves the interaction of cellular surface lipids, possibly cholesterol, or phospholipids with the surface lipids of HDL_3. A recent study in human liver membranes is in agreement with this hypothesis (Mendel et al., 1988). However, direct evidence that the HDL binding site is true receptor protein has been provided by ligand blotting analysis of crude membrane preparations from different cells and from human placenta. In this organ the dominant HDL binding protein has an approximate molecular mass of 80 kDa (Graham and Oram, 1987). Using a purified Apo A-I as ligand, Keso et al. (1987) identified a 120 kDa binding protein in the same placental preparations.

Biochemical investigations do not provide us

with any information on the topographic distribution of HDL_3 receptors and on the mechanism of an eventual ligand internalization. Moreover, in placenta which have a complex anatomical organization, morphological investigations are required to clearly identify the cells investigated. Consequently, an ultrastructural visualization of HDL_3 uptake by physiologically active syncytiotrophoblast in culture was performed. In our experimental conditions, after 3 days of culture the multinucleated cells exhibited typical ultrastructural features of trophoblasts. Using monoclonal antibodies, positive immunostaining was observed for cytokeratin, α and βhCG. An increasing level of hCG and a slight increase in progesterone level were measured in the culture media (Malassiné et al., 1990). All of these facts, as in other studies (Kliman et al., 1986; Kato and Braunstein, 1989; Ringler et al., 1989), lead us to believe that the multinucleated cells observed are syncytiotrophoblasts. Since Handley et al. (1981) described the first method of conjugating lipoprotein to colloidal gold, direct incubation of cells with gold lipoprotein has been used by authors for the study of lipoprotein uptake (Robeneck et al., 1984; Mommaas-Kienhuis et al., 1985; Paavola et al., 1985; Malassiné et al., 1987, 1990). In our experiments the adsorption of 4–6 lipoprotein particles to gold is similar to other findings (Schmitz et al., 1985). The competition experiments, which showed that bound HDL_3 conjugates were displaced in the presence of an excess of unlabelled HDL_3, assumed the specificity of the binding. The gold-HDL_3 particles bind at 4°C to the plasma membrane of syncytiotrophoblasts in culture with random distribution. In earlier studies, Malassiné et al. (1987–1990) found that binding sites for LDL and acetyl-LDL on human syncytiotrophoblasts show the same distribution pattern. However, when the syncytiotrophoblast in culture was exposed to gold-HDL_3 at 37°C for various times, the internalization process was not observed. The presence of gold-HDL_3 in endosomes of placental macrophages is similar to that reported in murine macrophages (Schmitz et al., 1985) assuming the assay validity. Our ultrastructural study in syncytiotrophoblasts is in agreement with biochemical data showing the absence of [^{125}I]HDL_3 degradation by mixed placental cells (Winkel et al., 1980) and by isolated trophoblast cells in suspension (Jorgensen et al., 1989). Thus, by both biochemical and ultrastructural investigations we demonstrate that HDL_3 specifically bind to syncytiotrophoblast surface without internalization. By similar methods Schmitz et al. (1985) demonstrated that HDL particles are bound and internalized by murine macrophages and then secreted by retroendocytosis without lysosomal degradation. Oram et al. (1987) suggested that HDL receptors facilitate cholesterol transport from cells by reversible binding of HDL to its cell-surface receptor, without subsequent internalization. Recently, Pittman et al. (1987) have suggested that at least two processes function simultaneously, one involving endocytotic uptake of Apo A-I (and thus presumably the HDL particle), and the other involving a direct transmembrane transport of the cholesterol ester moiety, without uptake of the particle.

The physiological significance of such an HDL_3 binding without internalization remains to be elucidated. An HDL pathway for cholesterol delivery to steroidogenic tissues has been described for certain species, such as rodents where HDL are the major plasma cholesterol carrier (Gwynne and Strauss, 1982). In addition to the reverse cholesterol transport function HDL may act directly on the membrane components after binding to a receptor either as a growth promoter or as a modulator of hormonal release. Thus, Darbon et al. (1986) demonstrated that HDL and more precisely its aproprotein moiety, stimulate phosphorylation of a 27 kDa protein in bovine endothelial cells, an effect mimicked by 12-*O*-tetradecanoylphorbol 13-acetate (TPA) and 1-oleoyl-2-acetylglycerol, both known to stimulate protein kinase C. Handwerger et al. (1987) reported that the apoprotein constituents of HDL (Apo A-I, Apo A-II and C-I) can stimulate the release of hPL from placental explants by a protein kinase C-dependent process (Sane et al., 1988). In human trophoblast cells, Wu et al. (1988) demonstrated an effect of HDL on adenylate cyclase activity and cAMP production, suggesting a role of cAMP as second messenger for hPL release. Recently, Jorgensen et al. (1989) showed that high affinity HDL_3 binding was not necessary for this biological function.

Our previous studies have demonstrated specific and distinct receptors for LDL and acetyl-LDL on isolated microvillous membranes and syncytiotrophoblasts in culture (Alsat et al., 1988). The internalization process was visualized for LDL and acetyl-LDL (Malassiné et al., 1987, 1990) suggesting the presence of a classical LDL endocytosis pathway and an additional 'scavenger receptor' endocytosis. This uptake of native and modified LDL could provide the cholesterol necessary for both placental steroidogenesis and intensive cellular growth. Whether HDL_3 are implied in the placental cholesterol supply as it has been described in human granulosa cells (Enk et al., 1987) needs further investigations.

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Relationship between inositol 1,4,5-trisphosphate mass level and [^{14}C]aminopyrine uptake in gastrin-stimulated parietal cells

Serge Roche, Tutus Gusdinar, Jean-Pierre Bali and Richard Magous

Laboratoire de Biochimie des Membranes, CNRS UPR-8402 – INSERM U-249, Faculté de Pharmacie, 34060 Montpellier Cedex, France

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Key words: Gastrin; Inositol 1,4,5-trisphosphate content; L-365,260; Acid secretion; Gastric parietal cell; [^{14}C]Aminopyrine uptake

Summary

The relationship between gastrin-stimulated inositol 1,4,5-trisphosphate (Ins(1,4,5)P_3) content and [^{14}C]aminopyrine ([^{14}C]AP) uptake (an index of in vitro acid secretion) was investigated in a population of highly enriched rabbit parietal cells (90 ± 5%). Gastrin induced a rapid rise in Ins(1,4,5)P_3 content which was maximal within 15 s of stimulation (2- to 2.5-fold basal level) followed by a rapid decrease within 30 s; a high Ins(1,4,5)P_3 level could also be observed after a longer time of hormone stimulation (180 s). Gastrin dose-dependently induced Ins(1,4,5)P_3 accumulation and [^{14}C]AP uptake; both dose–response curves were similar ($EC_{50} \approx 0.1$ nM). Furthermore, L-365,260 (3-(acylamino)benzodiazepine), a selective gastrin/CCK-B receptor antagonist, dose-dependently inhibited Ins(1,4,5)P_3 production and [^{14}C]AP accumulation induced by 10 nM gastrin with a similar potency ($IC_{50} \approx 1–2$ nM). These results led us to conclude that Ins(1,4,5)P_3 is involved in gastrin-stimulated acid secretory activity of gastric parietal cells.

Introduction

Regulation of HCl secretion is a complex mechanism involving neural, paracrine and endocrine processes and gastrin is the major secretagogue of the endocrine process. Although the mechanism whereby this hormone induces acid secretion is not clearly established, gastrin was described to stimulate acid secretion from an isolated population of gastric parietal cells (for review, see Soll and Berglindh, 1987).

In gastric parietal cells, gastrin-induced acid secretion is accompanied by a rise in free Ca^{2+} intracellular level (Muallem and Sachs, 1984; Tsunoda et al., 1988; Cabero et al., 1989). One of the earliest events of hormone-mediated Ca^{2+} mobilization is the activation of phosphoinositide breakdown which results in a rapid formation of the intracellular messengers, inositol 1,4,5-trisphosphate (Ins(1,4,5)P_3), leading to Ca^{2+} release from intracellular stores and *sn*-1,2-diacylglycerol, a strong activator of protein kinase C (for review, see Berridge, 1987). Several reports have shown that gastrin induces inositol phosphate production (Puurunen and Schwabe, 1987; Chiba et al., 1988; Roche and Magous, 1989) which parallels [^{14}C]aminopyrine ([^{14}C]AP) uptake (an index of in vitro acid secretion) into parietal cells (Chiba et al., 1988; Roche and Magous, 1989). Recently,

Address for correspondence: Dr. Richard Magous, Biochimie CNRS-INSERM, Faculté de Pharmacie, 15 avenue Charles Flahault, 34060 Montpellier Cedex, France.

using [^{3}H]-inositol-prelabelled parietal cells, we evidenced a rapid rise in [^{3}H]Ins(1,4,5)P_3 production in parietal cells maximally stimulated by gastrin (Roche and Magous, 1989).

Nevertheless, there is no direct evidence for a relationship between Ins(1,4,5)P_3 accumulation and gastrin-stimulated acid secretion: acid secretion could be triggered in a dose range where gastrin had no effect on Ins(1,4,5)P_3 production; in this way, CCK-8, known to enhance amylase release from pancreatic acini, increases inositol phosphate accumulation in these cells (for review, see Gardner and Jensen, 1987) and a recent report showed that CCK-8-induced amylase release was not related to Ins(1,4,5)P_3 production (Matozaki et al., 1990). Furthermore, the interpretation of Ins(1,4,5)P_3 measurements by the radiolabelling method in terms of Ins(1,4,5)P_3 mass is limited. In particular, it has to be assumed that there are no distinct pools of phospholipids (Monaco, 1982; Michell et al., 1988) or that the radioisotope is equally distributed throughout the different pools. Thus, it is unlikely that measurement of [^{3}H]Ins-(1,4,5)P_3 intracellular level could reflect the effective Ins(1,4,5)P_3 content within the cells.

Therefore, to elucidate whether or not Ins-(1,4,5)P_3 is involved in the acid secretory process induced by gastrin, we investigated, in this paper, the relationship between [^{14}C]AP uptake and Ins(1,4,5)P_3 mass level (measured by radioreceptor assay) following gastrin stimulation of a highly enriched parietal cells ($90 \pm 5\%$) population from rabbit gastric mucosa. Moreover, we studied the effects of L-365,260 (3-(acylamino)-benzodiazepine), a potent and selective CCK-B/gastrin receptor antagonist (Bock et al., 1989) on gastrin-induced Ins(1,4,5)P_3 accumulation and [^{14}C]AP uptake. This compound was previously shown to interact competitively with 'gastrin-type' receptors and to behave as an antagonist of gastrin-induced acid secretion in various animal models (Lotti and Chang, 1989).

Materials and methods

Unsulphated gastrin, (Ahx11)-HG-17, was a gift of Prof. E. Wünsch and Prof. L. Moroder (Max Planck Institut für Biochemie, Munich, F.R.G). L-365,260 was a gift of Dr. P. Anderson (Merck Sharp & Dohme, U.S.A). [^{14}C]Aminopyrine (0.1 Ci/mmol) and Ins(1,4,5)P_3 radioreceptor assay kit were from Amersham. Collagenase was from Serva (Heidelberg, F.R.G.). Nycodenz was from Accurate Chemical (U.S.A). Standard medium comprised Earle's balanced salt solution without bicarbonate containing 10 mM Hepes and 0.2% bovine serum albumin (pH 7.4). Earle's balanced salt solution was from Biomerieux (France).

Preparation of isolated rabbit gastric parietal cells

Cell isolation was carried out following the collagenase/ EDTA procedure as already described (Magous et al., 1989). Cell separation was performed by counterflow centrifugation with a Beckman elutriator rotor JE6-B. Elutriation fraction containing 70–75% parietal cells was layered onto Nycodenz gradient ($d = 1.04$–1.08 g/ml) according to the method of Chew et al. (1986). The top fraction of the gradient which contained 90% pure parietal cells ($\geq 90\%$ viability) was used for subsequent studies.

[^{14}C]Aminopyrine accumulation

H^+ secretion from parietal cells was determined using [^{14}C]AP accumulation as previously described (Roche and Magous, 1989). Cells (2×10^6 per ml) were incubated in standard medium with 0.05 μCi [^{14}C]AP with various concentrations of stimulants for 20 min at 37°C under continuous gassing (95% O_2/5% CO_2). [^{14}C]AP accumulation was measured by the radioactivity recovered in the cell pellet and expressed as a percentage of the radioactivity present in the incubation medium. Results are calculated as the difference between [^{14}C]AP accumulation in stimulated cells and [^{14}C]AP accumulation in unstimulated cells (Δ[^{14}C]AP uptake) (Roche et al., in press).

Ins(1,4,5)P_3 content measurement

Cells (4×10^6/ml) were preincubated for 5 min at 37°C with or without L-365,260 and then incubated with stimulants for various periods of time. The reaction was stopped by addition of perchloric acid 5% (w/v) final and the pH of the supernatant was adjusted to pH 7 by addition of 3 M KOH. 100 μl of the final supernatant was incubated for 15 min at 4°C with [^{3}H]Ins(1,4,5)P_3

($\approx$ 7 nCi) and with a specific Ins(1,4,5)P_3 binding protein. After centrifugation (12,000 $\times$ g for 3 min), the supernatant was removed and the pellet was assayed for radioactivity content. The amount of Ins(1,4,5)P_3 in the test sample was calculated from standard curve using authentic Ins(1,4,5)P_3.

Results

In rabbit parietal cells, basal Ins(1,4,5)P_3 content was 4.73 $\pm$ 0.47 pmol per 500,000 cells (mean $\pm$ SE, $n = 10$). As shown in Fig. 1, gastrin (10 nM) caused a rapid formation of Ins(1,4,5)P_3 which was maximal after 15 s of hormone stimulation and corresponded to 2- to 2.5-fold basal level. This stimulation rapidly decreased within 30 s but a high level in Ins(1,4,5)P_3 content (1.5- to 2-fold basal value) could be observed after a longer time of gastrin exposure (180 s).

As shown in Fig. 2, gastrin dose-dependently stimulated [^{14}C]AP accumulation and Ins(1,4,5)P_3 formation with a maximal effect observed for a $>$ 10 nM hormone concentration. Both dose–response curves were similar; concentrations that caused half-maximal stimulation were respectively: 0.085 $\pm$ 0.037 nM (Ins(1,4,5)P_3 formation) and 0.095 $\pm$ 0.025 nM ([^{14}C]AP uptake).

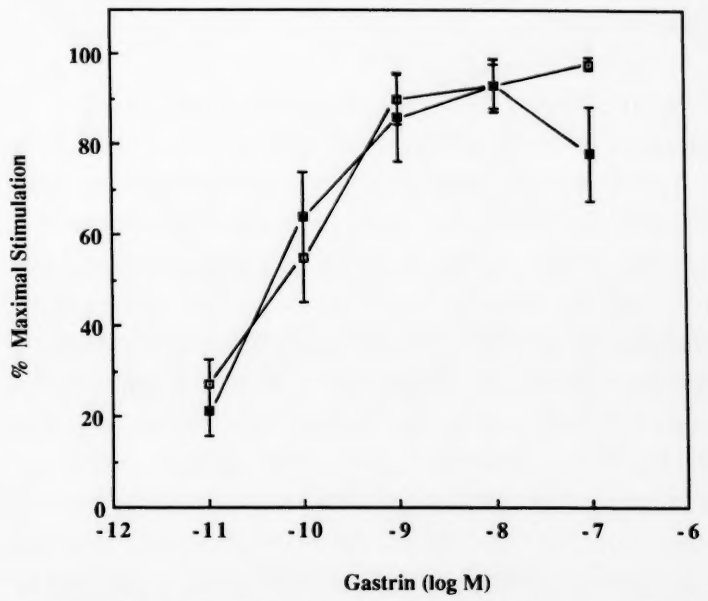

Fig. 2. Effects of gastrin on [^{14}C]aminopyrine accumulation and Ins(1,4,5)P_3 production in parietal cells. Cells were incubated with gastrin for 20 min at 37°C for measurement of [^{14}C]AP uptake (□) and for 15 s at 37°C for measurement of Ins(1,4,5)P_3 content (■). Results (mean $\pm$ SE from four separate experiments) are expressed as the percent of the maximal Δ[^{14}C]AP uptake or the maximal Ins(1,4,5)P_3 production caused by gastrin. Basal [^{14}C]AP accumulation was 5.4 $\pm$ 0.3%, maximal [^{14}C]AP accumulation induced by gastrin was 7.8 $\pm$ 0.6% and maximal Ins(1,4,5)P_3 content in gastrin-stimulated cells was 10.46 $\pm$ 0.84 pmol/500,000 cells.

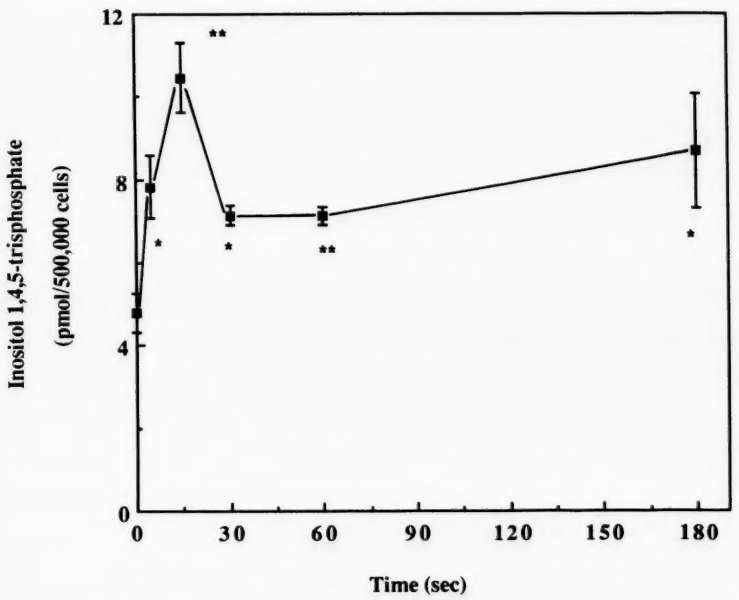

Fig. 1. Time-course of Ins(1,4,5)P_3 formation in gastrin-stimulated parietal cells. Parietal cells were incubated with 10 nM gastrin at 37°C for indicated times and Ins(1,4,5)P_3 content was quantitated as described in Materials and Methods. Results are means $\pm$ SE from four separate experiments. Data found in the presence of gastrin were compared with the zero-time point for statistical significance using Student's t-test: $^*P < 0.05$; $^{**}P < 0.01$.

The benzodiazepine analogue L-365,260 alone had no significant effect on basal [^{14}C]AP uptake and on Ins(1,4,5)P_3 level (not shown). L-365,260 inhibited in a dose-dependent manner [^{14}C]AP uptake and Ins(1,4,5)P_3 formation induced by 10 nM gastrin and a total inhibition was observed for a 100 nM L-365,260 concentration (Fig. 3). Furthermore, L-365,260 displayed a similar profile of action in inhibiting both events; the concentrations that caused half-maximal inhibition were respectively: 1.25 $\pm$ 0.33 nM (Ins(1,4,5)P_3 formation) and 2.5 $\pm$ 0.8 nM ([^{14}C]AP accumulation).

To elucidate whether or not L-365,260 competitively inhibited gastrin-induced Ins(1,4,5)P_3 production, the effect of a fixed concentration of L-365,260 (0.5 nM) was examined on Ins(1,4,5)P_3 formation stimulated by various concentrations of gastrin. As shown in Fig. 4, this compound caused a parallel rightward shift of this dose–response curve to gastrin.

Discussion

In the present study, we measured $Ins(1,4,5)P_3$ mass level in a highly enriched parietal cell preparation ($\approx 90\%$) from rabbit gastric mucosa, using the $[^3H]Ins(1,4,5)P_3$ assay system that contains a specific $Ins(1,4,5)P_3$ binding protein. This method allowed to determine the effective $Ins(1,4,5)P_3$ content within the cells and eliminated ambiguous results due to a contamination by inositol 1,3,4-trisphosphate and inositol 1,3,4,5-tetrakisphosphate. Our results confirm that gastrin induced a rapid rise in $Ins(1,4,5)P_3$ formation as previously observed with [^{3}H]inositol-prelabelled parietal cells. Some discrepancies could exist between the present results and those obtained with the [^{3}H]inositol-labelling method since maximal $[^3H]Ins(1,4,5)P_3$ accumulation occurred after 5–7 s of gastrin stimulation and rapidly decreased within 15 s (Roche and Magous, 1989): this difference could be due to the lack of equilibrium in phosphoinositide labelling since parietal cells were incubated in the presence of [^{3}H]inositol for a short period of time (2 h).

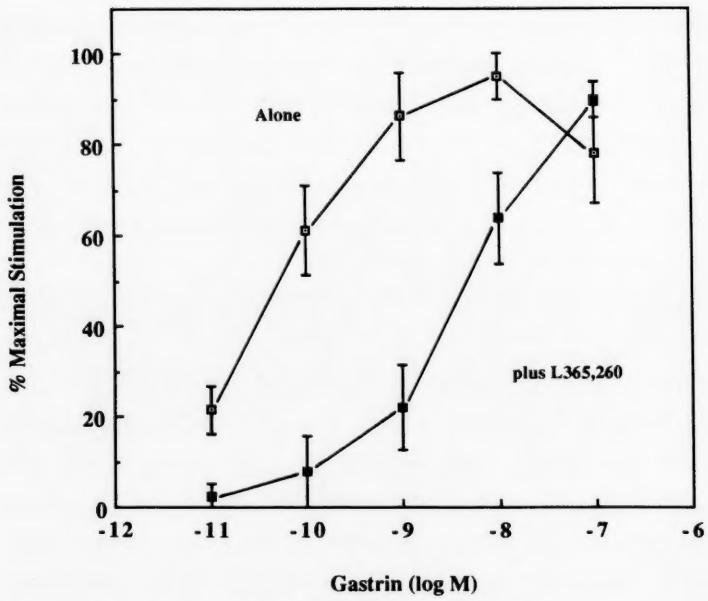

Fig. 4. Effect of L-365,260 on the dose–response curve for gastrin-stimulated $Ins(1,4,5)P_3$ production in parietal cells. Cells were preincubated for 5 min at 37°C without (□) or with (■) 0.5 nM L-365,260 and then incubated for 15 s at 37°C with the indicated concentrations of gastrin. Results (mean ± SE from four separate experiments) are expressed as the percent of the maximal $Ins(1,4,5)P_3$ production induced by gastrin.

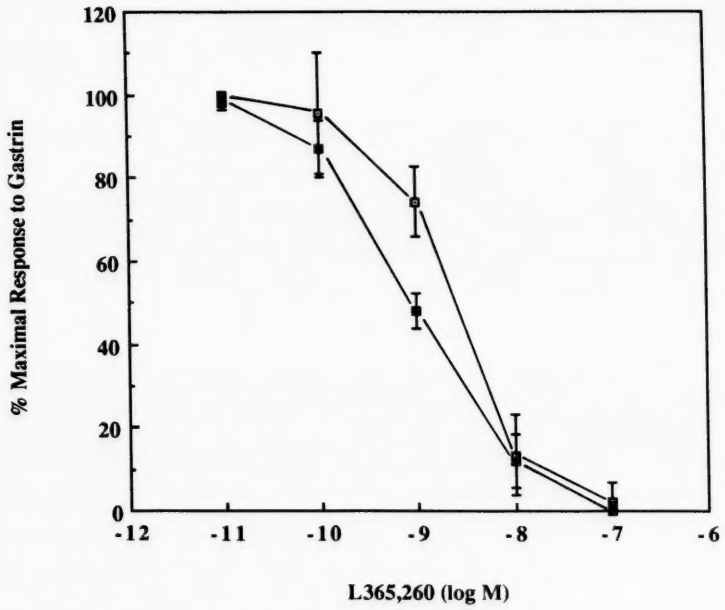

Fig. 3. Ability of L-365,260 to inhibit gastrin-induced $Ins(1,4,5)P_3$ formation and [^{14}C]aminopyrine uptake into parietal cells. Cells were preincubated for 5 min at 37°C without or with the indicated concentrations of L-365,260 and then incubated with 10 nM gastrin for 15 s at 37°C for measurement of $Ins(1,4,5)P_3$ mass (■). Cells were incubated for 20 min at 37°C for measurement of [^{14}C]AP uptake (□) without or with the indicated concentrations of L-365,260 and with 10 nM gastrin. Results (mean ± SE from five separate experiments) are the percent of Δ[^{14}C]AP uptake and of $Ins(1,4,5)P_3$ formation induced by 10 nM gastrin alone.

Gastrin exhibited the same profile of action in stimulating $Ins(1,4,5)P_3$ formation and [^{14}C]AP accumulation; the selective gastrin/CCK-B receptor antagonist L-365,260 induced a parallel rightward shift of the dose–response curve to gastrin for $Ins(1,4,5)P_3$ production, confirming that this compound behaves as a competitive inhibitor of gastrin effects. Furthermore, L-365,260 inhibited hormone-induced $Ins(1,4,5)P_3$ formation and [^{14}C]AP uptake in a parallel fashion. Taken together, these observations led us to conclude that $Ins(1,4,5)P_3$ is involved in the acid secretory process of parietal cells induced by gastrin. It is of some note that the IC_{50} value obtained for the inhibition of gastrin-induced $Ins(1,4,5)P_3$ accumulation was in good agreement with the IC_{50} value obtained for the inhibition of [^{125}I]gastrin binding to rabbit parietal cells (3.85 ± 0.5 nM) (Roche et al., in press) or to guinea-pig gastric glands (1.1 ± 0.4 nM) (Lotti and Chang, 1989).

$Ins(1,4,5)P_3$ formation following gastrin stimulation must be responsible at least in part for the Ca^{2+} fluxes observed in hormone-activated parietal cells since time-courses for $Ins(1,4,5)P_3$ pro-

duction and Ca^{2+} mobilization (Muallem and Sachs, 1984; Tsunoda et al., 1988; Cabero et al., 1989) fit in with the classical scheme of a primary phospholipase C stimulation which activates phosphoinositide breakdown and since experiments performed on permeabilized guinea-pig parietal cells demonstrated that this Ca^{2+} mobilization was due to Ins(1,4,5)P_3 (Tsunoda et al., 1988). However, these results do not exclude a possible role for other intracellular messengers in gastrin-stimulated parietal cells: in this way, we previously observed a rapid rise in [^{3}H]inositol 1,3,4,5-tetrakisphosphate accumulation in [^{3}H]inositol-prelabelled cells following hormone stimulation; as inositol tetrakisphosphate is known to affect the intracellular free Ca^{2+} concentration (Peterson, 1989; Joseph et al., 1989), it seems likely that this compound can play a role in Ca^{2+} fluxes induced by gastrin. Diacylglycerol formed following phosphoinositide hydrolysis can also be involved in the intracellular mediation of gastrin effects: a recent report showed that gastrin induced protein kinase C activation (Chiba et al., 1989). However, since the content of this inositol tetrakisphosphate and of diacylglycerol has not been determined in gastrin-stimulated cells, it cannot be concluded in favour of their possible involvement in the mediation of the hormone effects.

In conclusion, the present report demonstrated that gastrin induced, in a parallel fashion, an increase in Ins(1,4,5)P_3 mass level and [^{14}C]AP uptake into gastric parietal cells. The use of L-365,260, a specific antagonist of gastrin/CCK-B receptor-type, allowed us to evidence the involvement of Ins(1,4,5)P_3 in gastrin-stimulated acid secretory activity of parietal cells.

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Regulation of the expression of mitogen-regulated protein (MRP; proliferin) and cathepsin L in cultured cells and in the murine placenta

Marit Nilsen-Hamilton [1,3], Yng-Ju Jang [2], Michael Delgado [2], Jae-Kyoung Shim [2,3], Kerry Bruns [2], Chia-Ping Chiang [2], Yu Fang [1], Craig L.J. Parfett [4], David T. Denhardt [5] and Richard T. Hamilton [2,3]

Departments of [1] Biochemistry and Biophysics and [2] Zoology and [3] Molecular, Cellular and Developmental Biology Program, Iowa State University, Ames, IA 50011, U.S.A., [4] Environmental Health Center, Health and Welfare Canada, Tunney's Pasture, Ottawa, Ontario, Canada, and [5] Nelson Biological Laboratory, Rutgers, Piscataway, NJ 08855, U.S.A.

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Key words: Cathepsin L; Mitogen-regulated protein; Proliferin; Placenta, murine; Gestation; Growth factor

Summary

The genes encoding mitogen-regulated protein (MRP; also called proliferin; PLF) and procathepsin L (CL; also called major excreted protein; MEP) are expressed to high levels in the mouse placenta. Although they are both regulated by epidermal growth factor (EGF) and fibroblast growth factor (FGF) in 3T3 cells, expression of these genes is differently regulated with growth state. The expression patterns of MRP and CL as a function of murine development are also different. Basal and growth factor-stimulated levels of MRP expression are much higher in growing than in quiescent 3T3 cells, whereas CL levels are similar. These changes in gene expression in cultured quiescent cells parallel the changes in MRP and CL expression observed in the late-gestational quiescent placenta. These results suggest growth factors may regulate the expression of these genes, but other influences also regulate the expression of MRP and CL in vivo.

Address for correspondence: Marit Nilsen-Hamilton, Department of Biochemistry and Biophysics, Iowa State University, Ames, IA 50011, U.S.A.

Abbreviations: BSA, bovine serum Albumin; CS, calf serum; EGF, epidermal growth factor; FCS, fetal calf serum; FGF, fibroblast growth factor; PDGF, platelet-derived growth factor; CL, mouse cathepsin L (also called major excreted protein; MEP); MRP, mitogen-regulated protein (also called proliferin; PLF); SDS, sodium dodecyl sulfate; PMA, 12-*O*-tetradecanoylphorbol 13-acetate; TGF, transforming growth factor; SSPE, saline-sodium phosphate-EDTA buffer; SSC, saline-sodium citrate; IGF, insulin-like growth factor.

Introduction

Mitogen-regulated protein (MRP), also called proliferin, is a heterogeneously glycosylated protein (M_r 34,000) whose amino acid sequence is related to mouse prolactin (Nilsen-Hamilton et al., 1980; Linzer and Nathans, 1984; Parfett et al., 1985), giant cells in the murine placenta synthesize and secrete MRP (Lee et al., 1988; Nilsen-Hamilton et al., 1988). Epidermal growth factor (EGF), fibroblast growth factor (FGF), platelet-derived

growth factor and phorbol myristate acetate each stimulate 3T3 cells to synthesize MRP and its transcript (Nilsen-Hamilton et al., 1980, 1987; Linzer and Nathans, 1984; Parfett et al., 1985; Fienup et al., 1986). The level of MRP mRNA and protein expression also changes during development (Lee et al., 1988; Nilsen-Hamilton et al., 1988).

Procathepsin L (CL; M_r 39,000) was originally identified as the most abundant protein secreted by 3T3 cells transformed by Kirsten murine sarcoma virus (Gottesman, 1978). Since then, a correlation between expression of the oncogene *ras* and CL has been noted in mouse 3T3 cells which, when stably transformed by viruses that harbor the *ras* gene, or when stimulated by growth factors, frequently secrete large amounts of CL (Gottesman, 1978; Denhardt et al., 1986; Gal et al., 1986). We have found that cathepsin L is expressed in larger amounts in the trophoblastic giant cells of the fetal placenta than in other fetal or maternal cells. Trophoblastic giant cells are the only cells that have been found to express MRP as measured by immunocytochemistry and in situ hybridization (Lee et al., 1988; Nilsen-Hamilton et al., 1988).

Although many genes have been described that are regulated by growth factors in cultured cells, few genes have been examined to determine whether they are regulated by growth factors in vivo. MRP and CL are both major products of 3T3 cells in culture and trophoblastic giant cells in vivo. In 3T3 cells, both genes are regulated similarly by growth factors. Treatment with EGF, FGF, and PDGF results in increased expression of MRP and CL (Nilsen-Hamilton et al., 1980; Linzer and Nathans, 1984), whereas treatment with TGF-β results in decreased expression of MRP and CL (Chiang and Nilsen-Hamilton, 1986). The expression of these genes is also regulated during placental development as shown here and previously for MRP (Lee et al., 1988; Nilsen-Hamilton et al., 1988).

In this report, we examine the relationship between the expression of MRP and CL in the placenta for evidence of how these genes are regulated in vivo. We have found that, although the developmental profiles of the expression of these genes are similar during mid-gestation they differ in early and late gestation. We also discovered a striking correlation between the expression of MRP in the placenta and proliferation of the placenta. The same correlation was found with 3T3 cells in culture. From these observations we conclude that MRP and CL are probably regulated by growth factors in vivo but that additional factors also regulate their levels through mouse placental development.

Materials and methods

Materials

Murine EGF was purchased from Collaborative Research, PMA from Sigma, and human recombinant TGF-α from Gibco-BRL. [^{35}S]Methionine (1195–1500 Ci/mmol) and [α-^{32}P]dCTP (3000 Ci/mmol) were purchased from Amersham-Searle and New England Nuclear. The [^{35}S]-methionine was stored in liquid nitrogen. Mice (CF-1; Charles River, MA, U.S.A.) were housed in an environmentally controlled room in which the light was controlled to give 12 h of light and 12 h of darkness.

Purified MRP was used to produce anti-MRP antiserum in rabbits (Chiang and Nilsen-Hamilton, 1986). The preimmune serum was collected from the rabbits before they were injected with MRP. The antiserum was shown to selectively immunoprecipitate MRP from the medium of [^{35}S]methionine-labeled 3T3 cells. It also stained a protein of the correct molecular weight for MRP in Western blots of extracts of murine placentas.

The EGF, TGF-α and FGF stock solutions contained 0.5% BSA. The PMA was dissolved in dimethyl sulfoxide (DMSO). All treatments included the addition of BSA to 0.005%. When PMA was present then 0.1% DMSO was also present throughout. These concentrations of BSA and DMSO do not affect DNA synthesis or protein synthesis and secretion by 3T3 cells.

The mouse CL and MRP probes were isolated from a λgt11 library (Parfett et al., 1985; Denhardt et al., 1986). A full-length MRP cDNA clone isolated from the same library was also used in these studies. The rat ribosomal 18S RNA probe (pDF8) which encoded 0.9 kb of the middle portion of the rat 18S RNA (Torczynski et al., 1983) was contributed by Drs. R. Torczynski and H. Busch (Wadley Institutes of Molecular Medi-

cine, Dallas, TX, U.S.A.). The mouse glyceraldehyde phosphate dehydrogenase cDNA was a gift from Dr. P. Curtis (Wistar Institute, Philadelphia, PA, U.S.A.). The α-actin cDNA probe was a gift from Dr. A. Minty (Pasteur Institute). The cDNA (pAM91) was derived from a library of adult mouse skeletal muscle cDNA made from RNA enriched in α-actin mRNA (Minty et al., 1981). It recognizes both α- and β-actin mRNA.

Mating and collection of placental samples

Two male CF-1 mice and four female CF-1 mice were put in each cage, and every morning before 10:00 h the females were checked for a plug in the vaginal orifice. The day on which the plug was found was counted as day 0 of pregnancy. The mice were killed by cervical dislocation, and placentae were collected in the afternoon between 16:00 and 18:00 h or in the morning as indicated in the legend to each figure. After being separated from the fetus and uterine wall, each placenta was frozen in liquid nitrogen or in a slurry of semisolid 100% isopentane over liquid nitrogen. The frozen placentae were stored at $-70°C$.

Cell culture and [^{35}S]methionine labeling

Stock cultures of murine Swiss 3T3 cells were grown in Dulbecco-Vogt's modified Eagle's medium (DME) supplemented with 10% calf serum and maintained as described previously (Nilsen-Hamilton et al., 1980). For experiments with growing cells, cells were plated at a density of approximately 1.0×10^4 cells/cm^2 on multiwell plastic dishes in DME containing 10% calf serum. The experiments were started 2–3 days later when the cells had reached 50% confluence. For experiments with quiescent cells, the cells were plated in DME with 10% calf serum and grown until they were confluent. Then the medium was changed to fresh DME containing 2% calf serum. After 2–3 days, the cells became quiescent, and additions were made to the wells. 16–18 h after the additions to growing or quiescent cells, the cells were labeled with [^{35}S]methionine for 4 h as described previously (Nilsen-Hamilton and Hamilton, 1987). [^{35}S]Methionine-labeled proteins in the NP-40-soluble cell fraction or in the medium of labeled cells were immunoprecipitated with a final concentration of 1% (v/v) anti-MRP or 1% (v/v) anti-CL antisera as described previously (Chiang and Nilsen-Hamilton, 1986). The amount of radiolabel incorporated into individual secreted proteins was determined by densitometry of fluorograms obtained after resolution of the radiolabeled proteins in the medium and in the immunoprecipitates by gel electrophoresis on 7.5–15% polyacrylamide-SDS gels (Nilsen-Hamilton and Hamilton, 1987). The relative amounts of [^{35}S]methionine incorporated into each band was normalised to the amount of [^{35}S]methionine incorporated into cellular proteins. The latter value was determined by precipitating 5 μl samples of the NP40-soluble cell fraction with 10% (w/v) trichloroacetic acid (TCA) and scintillation spectroscopy.

Preparation of RNA

In the same experiments in which the cells were assayed for [^{35}S]methionine labeling, parallel cultures plated on 15 cm culture dishes were used to prepare RNA for analyzing the MRP and CL mRNA levels. Total RNA was prepared from frozen placentae as described by Davis et al. (1986) and analyzed by Northern blot after being resolved through 0.8% agarose gels containing 0.66 M formaldehyde and blotted onto nitrocellulose or derivatized nylon membranes (Zeta Probe, Bio-Rad). The blots were hybridized at 52°C in 50% formamide, 7% SDS, 1% polyethylene glycol 20,000, 2 times SSPE, 0.5% nonfat dried milk, 0.5 mg/ml salmon sperm DNA and with 60–100 ng of ^{32}P-labeled cDNA probe prepared by nick translation or random priming. Washes were carried out twice at 22°C with $0.2 \times$ SSC and twice at 68°C with $0.2 \times$ SSC, 0.1% SDS. Autoradiograms were made by exposing the membrane to X-ray film (Kodak) at $-70°C$. A linear relationship was established between the density of film exposure and the number of cpm in the protein bands. All densitometric measurements were made within this range.

Avidin-biotin-peroxidase immunohistochemical staining

Paraffin-embedded placentae were cut into 3 μm-thick sections. The sections were stained by using the avidin-biotin peroxidase cytochemical kit from Vector Laboratories and counterstained with Weigerts hematoxylin and eosin. The stained

sections were dehydrated, sealed with coverslips mounted with Permount, examined and photographed under a light microscope.

Results

Expression of MRP and CL mRNA in the mouse placenta as a function of gestation

The relative steady-state levels of MRP and CL mRNAs in the placenta were determined from day 7 to the 18th day of gestation by Northern and slot-blot analyses (Fig. 1). The relative levels of β-actin mRNA or 18S ribosomal RNA were also determined for each RNA preparation. The same profiles were obtained whether the data for CL and MRP mRNA were normalized to the amount of β-actin mRNA, 18S ribosomal RNA, poly A^+, or total RNA in the samples. Therefore the figure shows the relative amounts of CL and MRP mRNA as a proportion of the total RNA loaded on the gel. These results show that the proportions of both CL and MRP mRNA are highest on days 10 through 12 and decrease after day 12. Later in gestation (days 15 onwards), the proportion of CL mRNA increases again, whereas the MRP mRNA does not increase. Another difference in the developmental profiles of the expression of these two mRNAs is that, whereas MRP mRNA cannot be detected before day 9 (see inset to Fig. 1), CL

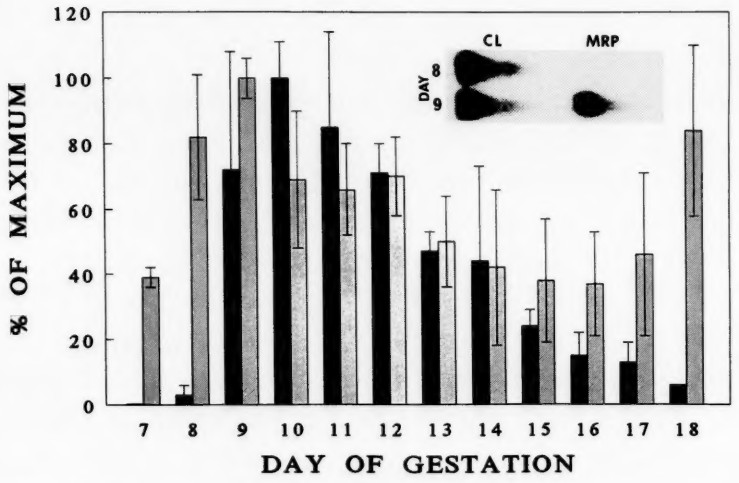

Fig. 1. Expression of MRP and CL in mouse placentae as a function of gestation. The relative amounts of mRNA were determined from densitometric scans of Northern and slot blots. The RNA preparations, RNA blots, and hybridization were done as described in Materials and Methods. The figure shows the average of four different sets of analyses. In each analysis, equal amounts of total or mRNA from placentae at different stages of development were loaded in each lane for each series tested. Each bar represents the average of 2–4 separate determinations on different preparations of placental RNA. The error bars show the standard deviations. The inset shows representative Northern blots of MRP and CL from placentas of days 8 and 9 in gestation. To clearly demonstrate the difference in level of MRP between day 8 and 9 the exposure of the film in the inset is higher than was used for quantitation. As described in Materials and Methods, all of the autoradiograms that were used for quantitation were exposed to the extent that they were within the linear range for quantitative densitometry. Black bars, MRP; grey bars, CL.

TABLE 1

EFFECT OF EGF, TGF-α AND PMA ON MRP AND CL PRODUCTION BY GROWING AND QUIESCENT CELLS

In experiment 1, quiescent and growing Swiss 3T3 cells were treated for 14.5 h with or without either 10 ng/ml EGF or 10 ng/ml PMA and then labeled for 4 h in the presence of the same factors with [^{35}S]methionine as described in Materials and Methods. In experiment 2, growing 3T3 cells were treated for 17 h with either 1 ng/ml FGF, 10 ng/ml EGF, or 4 ng/m1 TGF-α and then labeled with [^{35}S]methionine for 4 h as described for experiment 1. For both experiments, the radiolabeled proteins secreted into the medium were immunoprecipitated using polyclonal antibody directed against MRP or proCL. The immunoprecipitates were resolved by SDS-polyacrylamide gel electrophoresis and the area under the MRP or CL peaks determined by densitometry. The resulting areas were divided by the incorporation of protein by the cell layer to obtain the values shown in the table. In experiment 1 the average acid-insoluble cpm measured in 5 μl of cell lysate was 75,934 cpm for quiescent cells and 67,864 cpm for growing cells. Each value in the table represents the average of two independent samples followed by the standard deviation of the average.

Cells Treatment	MRP	CL
Experiment 1:		
Quiescent cells		
Control	4.2± 0.2	28.2± 4.3
EGF	32.5± 2.1	79.6±21
PMA	12.3± 0.1	57.8± 3.3
Growing cells		
Control	49.3± 5.9	13.4± 3.2
EGF	284.0±50	18.8± 1.7
PMA	201.4±38	10.7± 0.6
Experiment 2:		
Growing Cells		
Control	30.0± 4.4	8.2± 1.1
FGF	251.4±31	78.6±17.7
EGF	83.9±10	25.4± 5.0
TGF-α	102.4± 9.0	54.7±17.6

mRNA is abundant in the placenta even at this early time in placental development.

Relationship between placental growth and MRP production

MRP and CL were both detected in the placental trophoblastic giant cells by using avidin-biotin-peroxidase-linked immunocytochemical stains (Fig. 2). However, the distributions of the two proteins in the placental giant cells as detected by the antisera were different. Under higher-power lenses than shown in Fig. 2, anti-MRP antibody was seen bound to granular structures in a perinuclear arrangement, whereas the anti-CL antibody stain was more evenly distributed through the cytoplasm.

An analysis of the percentage of giant cells that contained CL and MRP as a function of development showed that the percentage of cells that expressed MRP dropped considerably after day 13, whereas the percentage of cells expressing CL remained fairly steady through gestation (Fig. 3). This profile can be compared with the profile of mRNA expression as a function of development (Fig. 1). The large drop in the percentage of giant cells in the placenta that contain MRP was corre-

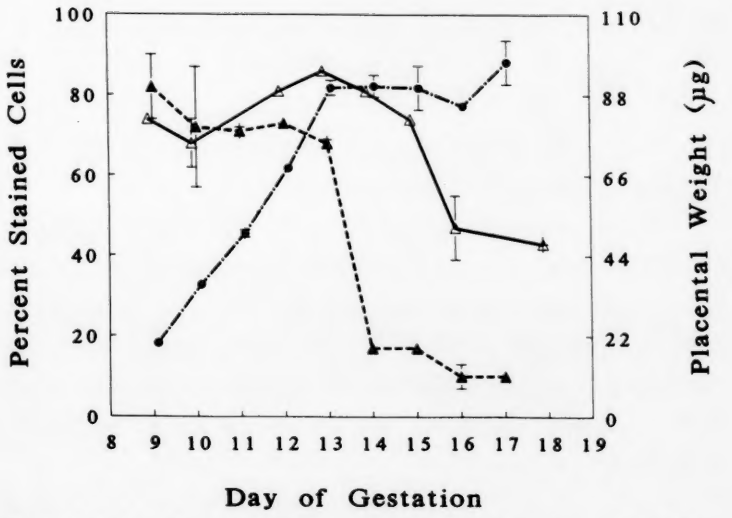

Fig. 3. Expression of MRP and CL as a function of developmental age. Placental slices were immunostained by using anti-MRP or anti-CL antibodies. The giant cells were identified by their large size. Approximately 200 giant cells were counted per section and it was determined whether each cell as stained with the antibody. The percentage of giant cells that expressed CL and MRP was determined at each stage of development. The results are plotted as a function of the number of days postcoitus, with the day of the plug being day 0. Two to four different placental slices were counted to obtain each value. The average weight of placentae at each developmental stage was determined. Two to three placentae were pooled and weighed together. Two such estimates were made to obtain each value. The average weights are presented. Filled triangles, MRP; open triangles, CL; filled circles, placental weight.

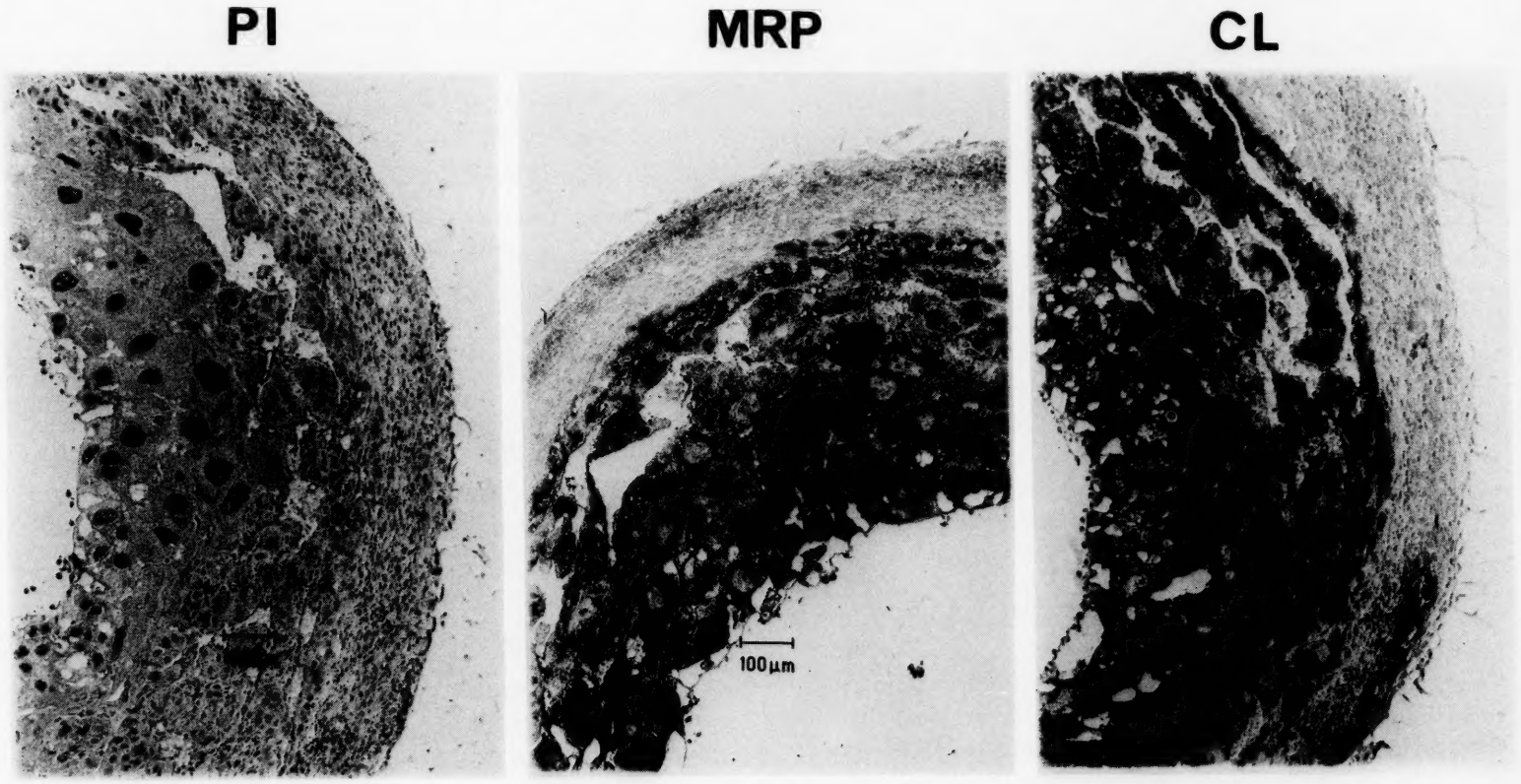

Fig. 2. Comparison of the distribution of MRP and CL in mouse placentae as visualised by immunoperoxidase staining. PI, preimmune serum; MRP, mitogen-regulated protein; CL, cathepsin L.

lated with the time when the placenta stopped increasing in size (Fig. 3).

The effect of FGF on the production of MRP and CL by growing vs. quiescent 3T3 cells

The expression of MRP and CL was examined in 3T3 cells as a function of growth state. Basal levels of MRP were higher in growing compared with quiescent cells (Fig. 4), and treatment with fibroblast growth factor resulted in much more MRP being secreted by growing cells than by quiescent cells.

As for MRP, fibroblast growth factor increased the production of CL in growing and in quiescent 3T3 cells. However, whereas expression of basal and induced MRP declined as the cells became quiescent, the basal amount of CL produced increased slightly. There was also a decrease in the induced level of CL in quiescent compared with growing cells. Similar results were found for cells stimulated by EGF or PMA (Table 1).

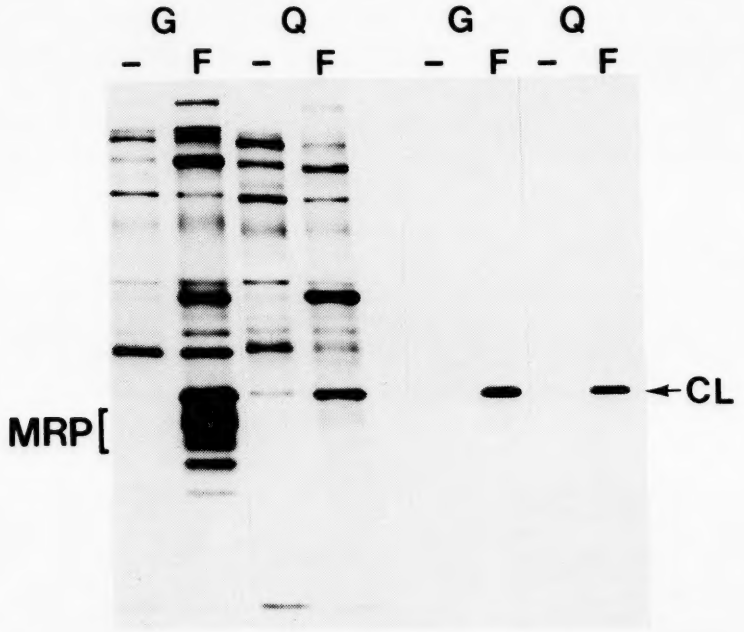

Fig. 4. The expression of MRP and CL by quiescent and growing 3T3 cells. Growing (G) and quiescent (Q) cells were stimulated with FGF (F) for 14.5 h then labeled with [^{35}S]methionine. Proteins secreted by the cells were identified after gel electrophoresis followed by fluorography. Control cells (−) were treated with 0.01% BSA which was also added with the FGF. The same [^{35}S]methionine labeling medium was used for both growing and quiescent cells in each experiment. The samples of medium were also immunoprecipitated by using anti-CL antiserum. The left panel shows all of the [^{35}S]methionine-labeled protein in each sample. MRP is clearly visible in these samples. The right panel shows the immunoprecipitated CL from each sample. The positions of MRP and CL are indicated.

Discussion

Synthesis of both MRP and CL is selectively increased in 3T3 cells stimulated by growth factors such as EGF, FGF, and PDGF (Nilsen-Hamilton et al., 1980, 1981; Linzer and Nathans, 1984) and by tumor promoters (Fienup et al., 1986). The molecular basis for these increases probably is an increase in the rate of transcriptional initiation from the genes encoding these proteins (Rabin et al., 1986; Linzer and Mordacq, 1987). Transforming growth factor type beta (TGF-β) counteracts the stimulatory effects on MRP and CL synthesis (Chiang and Nilsen-Hamilton, 1986).

Here, we show that MRP and CL are found in the same cells in vivo. These are the trophoblastic giant cells of the fetal placenta. Because we had shown that MRP and CL expressions are regulated in parallel by growth factors in cultured cells (Nilsen-Hamilton et al., 1980), we tested the hypothesis that the expression of these genes is also regulated in parallel in vivo. To do this, we compared the developmental profiles of the expression of these two proteins and their mRNAs. If growth factors were the only regulators of the expression of these two genes, then we would expect identical developmental profiles. We found that, although there were some similarities in the developmental profiles of these two genes, there were clear differences. The similarities between the two developmental profiles occurred in mid-gestation when the placenta was growing and the amounts of both mRNAs were at their highest. Also in this period, about 70–80% of the giant cells contained MRP and CL. The differences in the developmental profiles of MRP and CL were seen at either end of the period of placental development.

The expression of MRP in the placenta correlates with the period of rapid placental growth. The first day after cessation of placental growth marks the day on which there is a large decrease in the percentage of giant cells that express MRP. This correlation is exactly reflected in the regulation of MRP expression in cultured 3T3 cells where the level of expression of MRP is high in growing cells and low in quiescent cells. The

placental giant cells are nonproliferating differentiated cells. Thus, it seems that one factor that might regulate the expression of MRP gene expression in the placenta and in cultured cells is the growth state of the surrounding cells.

Although the results of these studies show a correlation between proliferation and MRP gene expression, they do not provide evidence that cell proliferation regulates the production of MRP, or vice versa. The correlation between proliferation and MRP production in vivo and in cell culture could be fortuitous. For example, MRP expression and placental proliferation in the placenta may be regulated by the same growth factor. TGF-α is a likely candidate for a positive regulator of MRP production in the placenta. TGF-α is expressed maximally in the rat decidua on day 8 (Han et al., 1987) and has also been found in the mouse placenta (Wilcox and Derynck, 1988). Placental giant cells express the EGF receptor (Adamson et al., 1981). Here we show that TGF-α stimulates MRP and CL expression in 3T3 cells.

It has been suggested that MRP itself regulates cell proliferation (Nilsen-Hamilton et al., 1980; Linzer and Nathans, 1984). This idea is supported by the observation that expression of one form of MRP (PLF1) in a premyoblast cell line inhibits the expression of muscle-specific genes (Wilder and Linzer, 1989). However, our previous results suggest that MRP is not a proliferation factor for 3T3 cells. First, the presence of polyclonal, anti-MRP IgG in the tissue culture medium of 3T3 cells has no effect on the increase in DNA synthesis induced in 3T3 cells by growth factors (Fienup, 1985). Second, a clone of BALB/c 3T3 cells that does not synthesize MRP (Nilsen-Hamilton et al., 1981) is as effectively stimulated to proliferate by growth factors as is a clone of BALB/c 3T3 cells that makes and secretes the protein in response to growth factors (M.-H. Jeng and M. Nilsen-Hamilton, unpublished). Furthermore, although insulin, IGF-I, and IGF-II do not stimulate the production of MRP, they do stimulate DNA synthesis in 3T3 cells (L. Bendickson and M. Nilsen-Hamilton, unpublished).

In summary, we have compared the regulation of MRP and CL expression in 3T3 cells and the placenta. Although there were similarities in the regulation of these two genes in vivo and in cell culture, there were also clear differences. From our results, we propose that growth factors regulate the expression of MRP and CL in vivo, but that other influences are superimposed to alter the developmental profiles of these two genes. One factor that may have a strong influence on the level of gene expression in vivo is the growth state of the surrounding cells.

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A selective biotinylated probe for V_{1a} vasopressin receptors

John Howl, Ian D. Kerr *, Conrad H.W. Chan and Mark Wheatley

School of Biochemistry, University of Birmingham, Edgbaston, Birmingham, B15 2TT, U.K.

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Key words: Biotin; Vasopressin; V_{1a} receptor

Summary

We have designed and synthesized a biotinylated vasopressin antagonist which is a selective probe for studying the V_{1a} subtype of vasopressin receptor. Initially we synthesized the novel vasopressin analogue $d(CH_2)_5Tyr(Me)^2LysNH_2^9AVP$ (ALVP). Biotinamidocaproate was subsequently coupled to the ϵ-amino group of ALVP to generate the novel biotinylated probe $d(CH_2)_5Tyr(Me)^2Lys(N\epsilon$-biotinamidocaproate)$NH_2^9AVP$ (ALBtnVP). Pharmacological characterization of ALVP and ALBtnVP established that both ligands were high affinity antagonists at V_{1a} receptors, and that both displayed marked V_{1a}/V_2 selectivity. The observation that receptor-bound ALBtnVP was bi-functional, and thereby able to bind conjugated derivatives of avidin or streptavidin, allowed ALBtnVP to be utilized as a selective probe for V_{1a} receptors.

This strategy allowed the visualization of V_{1a} receptors on the surface of WRK-1 cells and hippocampal neurons, by using streptavidin-gold with electron microscopy and fluorescein-avidin with light microscopy. We conclude that ALBtnVP is a useful probe for V_{1a} receptors.

Address for correspondence: Dr. Mark Wheatley, School of Biochemistry, University of Birmingham, Edgbaston, Birmingham, B15 2TT, U.K.

* Present address, Department of Zoology, University of Nottingham, U.K.

Abbreviations: Boc, butyloxycarbonyl; DCC, *N,N'*-dicyclohexylcarbodiimide; DMF, dimethylformamide; Fmoc, 9-fluorenylmethoxycarbonyl; HOBT, *N*-hydroxybenzotriazole; OPfp, pentafluorophenyl ester; TEM, transmission electron microscopy; TFA, trifluoroacetic acid. Ligands: AVP, [8-arginine]vasopressin; $d(CH_2)_5Tyr(Me)^2AVP$, [1-β-mercapto-β,β-cyclopentamethylenepropionic acid, 2-*O*-methyltyrosine, 8-arginine]vasopressin; dVDAVP, 1-deamino-[4-valine, 8-D-arginine]vasopressin; ALVP, [1-β-mercapto-β,β-cyclopentamethylenepropionic acid, 2-*O*-methyltyrosine, 8-arginine, 9-lysinamide]vasopressin; ALBtnVP, [1-β-mercapto-β,β-cyclopentamethylenepropionic acid, 2-*O*-methyltyrosine, 8-arginine, 9-lysinamide(*N*ϵ-biotinamidocaproate)]vasopressin; Thr^4Gly^7-OT, [4-threonine, 7-glycine)oxytocin.

Introduction

In recent years it has been shown that receptors for the neurohypophysial hormone $[Arg^8]$vasopressin (AVP) are expressed in a variety of tissues, and that AVP regulates a diverse range of cellular functions in mammals. In common with many hormones and neurotransmitters, multiple subtypes of AVP receptor (VPR) have been delineated. Thus, the V_{1a} isoreceptor, which mediates the vasopressor action of AVP (Michell et al., 1979), is functionally coupled to phosphoinositidase C. V_{1a} receptors are expressed by vascular smooth muscle (Penit et al., 1983), hepatocytes (Cantau et al., 1980) and a rat mammary tumour cell line WRK-1 (Guillon et al., 1986). The V_2

receptor subtype stimulates adenylate cyclase and mediates the antidiuretic action of AVP in the kidney tubule (Jard et al., 1975; Michell et al., 1979). More recently, the pharmacological profile of a third VPR subtype has been described in anterior pituitary (Antoni, 1984; Baertschi and Friedli, 1985) which has been designated V_{1b} (Jard et al., 1986) to distinguish it from the V_{1a} subtype.

Studies of the function and pharmacology of VPR subtypes have benefitted greatly from the development, particularly by Manning's group (e.g. Manning et al., 1987), of a range of AVP analogues with enhanced receptor specificity. One such ligand is $d(CH_2)_5Tyr(Me)^2AVP$ (Kruszynski et al., 1980), a high affinity and highly selective V_{1a} receptor antagonist. Design of new antagonists has been assisted by the observation (Manning et al., 1984) that substitution, or deletion of the carboxy-terminal glycine, or glycinamide, residue in a range of AVP antagonists has little effect upon antagonistic potency. A biotinylated VPR antagonist which maintains high affinity, and high selectivity for V_{1a} receptors would be a versatile probe. The very high affinity binding between biotin and avidin or streptavidin has enabled the localization or isolation of many low abundance molecules (for a review see Wilchek and Bayer, 1988). Consequently, a biotinylated V_{1a}-selective ligand could be used in concert with avidin conjugated to electron-dense or fluorescent markers to visualize V_{1a} receptors. In addition, a biotinylated ligand could be used to develop a biospecific affinity column based upon biotin-avidin complex formation.

In this paper we describe the synthesis of a biotinylated antagonist which exhibits high affinity for V_{1a} receptor and possesses marked V_{1a}/V_2 selectivity. Furthermore, we confirm the bi-functional nature of the ligand by using it to visualize V_{1a} receptors on the surface of cells using avidin/streptavidin coupled to different reporter groups.

Materials and methods

[Phe-3,4,5,^{3}H]AVP 67.7Ci/mmol and the labelled V_{1a}-selective vasopressin antagonist [Phe-3,4,5, ^{3}H]$d(CH_2)_5Tyr(Me)^2AVP$ (Kruszynski et al., 1980) 56.2 Ci/mmol were from NEN. Unlabelled peptides AVP, $d(CH_2)_5Tyr(Me)^2AVP$ and the selective oxytocin agonist Thr^4Gly^7OT (Lowbridge et al., 1977) were supplied by Bachem. The V_2-selective agonist dVDAVP (Manning et al., 1973) was from Penninsula Laboratories. *N*-α-Boc-protected amino acids, β-(s-p-methoxybenzyl)-mercaptopentamethylene-propionic acid and *N*-α-Fmoc-Lys(Boc)-OPfp were from Novabiochem. Ultrosyn C resin was from Pharmacia. Biotinamidocaproate *N*-hydroxysuccinimide (NHS) ester and streptavidin-gold (mean particle diameter 18.3 nm) were from Sigma. Fluorescein avidin DCS (cell sorting grade) was from Vector Laboratories.

High performance liquid chromatography

Analytical HPLC analysis of peptides was performed using a 30 cm spherisorb C_{18} reverse phase column (Phase Sep, Deeside, U.K.). Unless otherwise stated, quoted elution times of peptides refer to their time of elution from the above column using a 30 min linear gradient of 5–65% acetonitrile/0.1% trifluoracetic acid (TFA), flow rate 1.5 ml/min with absorbance measured at 220 nm. Large scale purification of peptides was performed using a Vydac C_{18} preparative column.

Synthesis of $d(CH_2)_5Tyr(Me)^2LysNH_2^9AVP$ (ALVP)

Two methods have been employed. The products of each synthesis gave identical results.

Scheme 1. 20–50 μg of $d(CH_2)_5Tyr(Me)^2AVP$ (HPLC elution time 21:29 min) containing ca. 0.1 μCi of [^{3}H]$d(CH_2)_5Tyr(Me)^2AVP$ was dissolved in 500 μl of buffer A (100 mM Tris, 20 mM $CaCl_2$, pH 7.9) containing 8 μg of TPCK-treated trypsin (Worthington). Following incubation overnight at 30°C $desGlyNH_2^9d(CH_2)_5Tyr(Me)^2AVP$ was isolated by HPLC (elution time 22:57 min, yield 80–95%). The loss of terminal glycinamide residue, following trypsin digestion, was confirmed by amino acid analysis.

Ultrosyn C beads, preswollen in DMF, were deprotected with 20% piperidine/DMF for 10 min, washed with DMF, and loaded with 3 molar equivalents of Fmoc-Lys-(Boc)-OPfp + 3 molar equivalents of HOBT in DMF. The mixture was stirred overnight and beads washed with DMF and dried from ether. *N*-α-Fmoc was cleaved from lysine residues with 20% piperidine and $desGlyNH_2^9d(CH_2)_5Tyr(Me)^2AVP$ added with 3 molar equivalents of HOBT in the ratio of 10 mg

beads/50 μg peptide (ca. 100-fold excess lysine). DCC was added (3 molar equivalents) and the reaction allowed to proceed for 16–24 h. Beads were washed with DMF, dried from ether, and treated with 95% TFA to yield the fully deprotected peptide AVLP (Fig. 1) which was purified by HPLC (elution time 22:08 min, yield 6–15%).

Scheme 2. Uncyclized ALVP was prepared by solid phase synthesis by Dr. J. Fox (Alta Bioscience) and the amino acid content confirmed by amino acid analysis. Intramolecular disulphide bond formation was achieved by air oxidation. The cyclized peptide was lyophilized and purified by HPLC (elution time 22:08 min, yield 50–90%). Fast atom bombardment (FAB) mass spectrum analysis of the purified material confirmed the molecular weight of ALVP ($MH^+ = 1222.6$).

Synthesis of $d(CH_2)_5Tyr(Me)^2Lys(N\epsilon$-biotinamidocaproate)$NH_2^9AVP$ (ALBtnVP)

ALVP was mixed with a 10-fold molar excess of biotinamidocaproate NHS ester in DMF and incubated overnight. C_{18} reverse phase HPLC revealed two overlapping peaks corresponding to the precursor ALVP (elution time 22:08 min) and the product ALBtnVP (elution time 22:11 min, Fig. 1). However, the peptides were resolved by using a Lichrospher C_8 column (Merck) and a 30 min linear gradient of 5–65% acetonitrile/0.1% TFA with elution times of 10:22 min and 12:49 min for ALVP and ALBtnVP respectively. ALBtnVP was collected (yield ca. 30%), lyophilized, and the presence of biotin demonstrated by the method of McCormick and Roth (1970). The molecular weight of ALBtnVP was confirmed by FAB mass spectrometry ($MH^+ = 1562.1$).

Liver and kidney membrane preparation

Adult Wistar rats of both sexes were obtained from the University of Birmingham animal house. A partially purified rat liver membrane preparation was made following the method of Dickey et al. (1987) with minor modifications. Briefly, liver was homogenized in 4 volumes of ice-cold buffer B (20 mM Hepes; 10 mM $Mg(CH_3COO)_2$; 1 mM EGTA; 0.25 M sucrose; 1 mg/ml bacitracin, pH 7.4) supplemented with leupeptin and antipain (5 μg/ml) for the homogenization step only. The homogenate was centrifuged at $8000 \times g$ for 10 min at 4°C, the pellet discarded and the supernatant centrifuged further ($70,000 \times g$ for 60 min at 4°C). At this stage the upper microsomal layer was separated from the more dense glycogen pellet and resuspended by homogenization in fresh buffer B. A crude membrane fraction was sedimented at $40,000 \times g$ for 60 min and resuspended at a concentration of 1 g wet weight original tissue/2 ml buffer.

A membrane preparation of bovine kidney medulla was prepared using a modification of the method described by Bockaert et al. (1973). Bovine kidneys were obtained from a local abattoir and transported on ice. Medullary tissue was dissected at 0°C, minced with scissors, and washed with cold (4°C) buffer C (5 mM Tris, 0.25 M sucrose; 3 mM $Mg(CH_3COO)_2$; 1 mM EGTA; 1 mg/ml bacitracin, pH 7.4). Tissue (ca. 100 g) was homogenized in 200 ml of buffer C at 4°C and filtered through nylon gauze. The filtrate was centrifuged ($1500 \times g$, 20 min, 4°C) and the upper membrane pellet resuspended in 100 ml fresh buffer C. The preparation was maintained at 4°C and the process of centrifugation and resuspension repeated 6 times before a final resuspension in buffer B at a concentration of 2 g original wet weight of tissue/ml buffer.

All membrane preparations were stored in 1 ml aliquots at −20°C for up to 6 months with no detectable loss of binding capacity. The protein content of membranes was determined using the Pierce BCA assay using BSA (Sigma) as standard and ranged from 2–6 mg/ml total protein.

Ligand binding assays

Membranes were thawed, diluted in buffer D (20 mM Hepes; 10 mM $Mg(CH_3COO)_2$; 1 mM EGTA; 1 mg/ml BSA, pH 7.4), and then added to [^{3}H]ligand and unlabelled competing ligand at the concentrations indicated to give a final volume of 500 μl containing 100 μg of membrane protein. Binding incubations were for 90 min at 30°C which allowed equilibrium to be established. Free and bound [^{3}H]ligand were separated by centrifugation for 5 min at $10,000 \times g$. After superficial washing, the membrane pellet was solubilized by Soluene 350 (Packard) mixed with scintillation cocktail and counted by liquid scintillation spectroscopy. [^{3}H]$d(CH_2)_5Tyr(Me)^2AVP$ was used as

the tracer ligand for the rat liver V_{1a} receptor ($K_d = 0.28$ nM) and [^{3}H]AVP for the bovine kidney V_2 receptor ($K_d = 0.68$ nM).

IC_{50} values derived from competition binding experiments were corrected for occupancy by tracer ligand according to the method of Cheng and Prusoff (1973).

Visualization of WRK-1 cell V_{1a} receptors

WRK-1, a cloned cell line derived from a rat mammary tumour, which expresses receptors of the V_{1a} subtype (Guillon et al., 1986; Kirk et al., 1986) was maintained as previously described (Wong et al., 1988). Cells were grown to confluence and replated into 35 mm dishes, some containing colloidin coated electron microscope grids (Taub G-200), in 1.5 ml of culture medium. After 3 days cells were incubated with 20 nM ALBtnVP for 60 min at 37°C. Control incubations contained no biotinylated ligand or biotinylated ligand together with 10 μM d$(CH_2)_5$Tyr-(Me)^{2}AVP.

Cells grown on electron microscope grids were incubated with gold-labelled streptavidin (streptavidin-Au; 50 × dilution of stock, Sigma) for a further 4 h in culture medium. Grids were washed, fixed in 2.5% (v/v) glutaraldehyde (4°C overnight), and processed for TEM by dehydration, air drying, and negative staining. Cells were viewed on a Joel 1200 EX electron microscope operated at 80 kV.

For sectioning, confluent cell layers were incubated with ligands as detailed above, washed, and fixed in 1% (v/v) glutaraldehyde for 60 min at 37°C. Cells were scraped from the dish with a rubber policeman, washed, resuspended in 0.1 M cacodylate buffer pH 7.4 containing a 50 × dilution of the supplied stock streptavidin-Au, and incubated at 4°C for 4 h with periodic inversion. Cells were sedimented (10,000 × g, 5 min) and resuspended twice in 2.5% (v/v) glutaraldehyde/0.1 M cacodylate. Pelleted cells were processed for TEM by fixing in 1% (w/v) osmium tetroxide (60 min), dehydration through a graded alcohol series, and embedding in Epon. Sections were cut at 80–150 nm, stained in uranyl acetate and lead citrate and viewed on a Philips 301 electron microscope operated at 80 kV.

Visualization of vasopressin receptors in hippocampal slices

Hippocampal slices ca. 450 μm were cut from the brains of young adult Wistar rats and maintained in buffer E (0.15 M NaCl; 5 mM KCl; 22 mM $NaHCO_3$; 20 mM $MgSO_4$; 10 mM glucose; 1.25 mM Na_2HPO_4; 2 mM $CaCl_2$; 1 mg/ml bacitracin; 1 mg/ml BSA, pH 7.4) equilibrated with 95% O_2/5% CO_2. Individual slices were incubated in a capped vial in a volume of 2 ml buffer E at 30°C for 60 min with 20 nM ALBtnVP. Control incubations were performed either without biotinylated ligand or with biotinylated ligand together with either 5 μM d$(CH_2)_5$Tyr-(Me)^{2}AVP or 1 μM Thr^{4}Gly^{7}OT. After initial incubation, fluoroscein labelled avidin (Vector, DCS cell sorting grade) was added to a final concentration of 50 μg/ml and slices incubated for a further 30 min in the dark. Slices were fixed by the addition of formaldehyde (final concentration 2.5% (w/v)) at 4°C in the dark for 60 min. Specimens were washed briefly and mounted in glycergel (Dako). Slices were viewed using epifluorescent illumination on a Leitz Ortholux microscope.

Assay of glycogen phosphorylase a in hepatocytes

Rat hepatocytes were prepared from the liver of adult Wistar rats using the two-step collagenase method of McGowan et al. (1981) except that the perfusion medium was a Ca^{2+}-, Mg^{2+}-free Krebs-Ringer bicarbonate buffer with Hepes (10 mM), pH 7.4. The protocol used for the hormonal stimulation of glycogen phosphorylase (EC 2.4.1.1) was based upon the method of Kirk et al. (1979). Hormonal activation of glycogen phosphorylase was for 2 min at 37°C. Subsequently, glycogen phosphorylase *a* activity was assayed by measuring the incorporation of radioactive [^{14}C]glucose-1-phosphate into glycogen at 30°C (Hems et al., 1976).

Results

Pharmacological characterization of ALVP and ALBtnVP binding to V_{1a} and V_2 receptors

The ligands ALVP and ALBtnVP (Fig. 1) were synthesized as described in Materials and Methods. Competition experiments were undertaken in

order to determine the affinity of ALVP and ALBtnVP for both the V_{1a} receptor and the V_2 receptor expressed by rat liver and bovine kidney medulla respectively. Specific binding of [^{3}H]d-$(CH_2)_5$Tyr(Me)^{2}AVP (0.125 nM) to bovine kidney medulla membranes was not detected confirming an absence of V_{1a} receptors in this preparation. Analysis by HPLC revealed that the ligands used were stable and resistant to proteolysis in the presence of liver or kidney membranes for at least 48 h under the incubation conditions used. Competition binding curves for each ligand, together with those of AVP and its V_2-selective analogue dVDAVP, are shown in Fig. 2. In rat liver, both ALVP and ALBtnVP competed with labelled tracer ligand [^{3}H]d$(CH_2)_5$Tyr(Me)^{2}AVP with high affinity (Table 1 and Fig. 2*a*). In contrast, both ALVP and ALBtnVP exhibited a relatively low affinity for the V_2 receptor (Table 1 and Fig. 2*b*). It can be seen from the data presented in Table 1 that both ALVP and ALBtnVP exhibited marked V_{1a}/V_2 selectivity. In addition, ALVP was demonstrated to be an antagonist in functional studies using AVP-stimulated glycogen phosphorylase *a* activity in freshly prepared hepatocytes. AVP (1 nM) stimulated glycogen phosphorylase *a* from

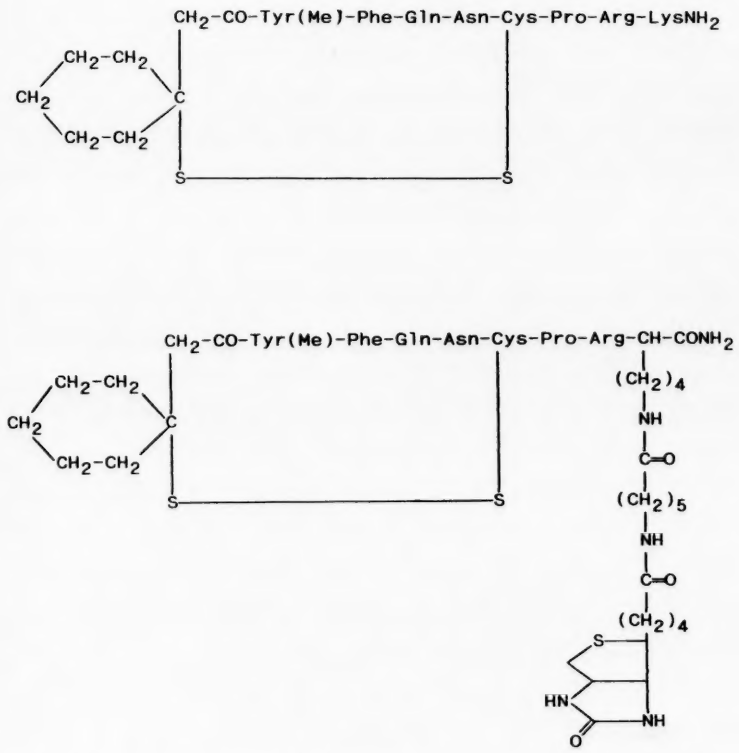

Fig. 1. Structural formulae of ALVP (top) and its biotinylated analogue ALBtnVP (bottom). The full names of these peptides are given in the list of abbreviations.

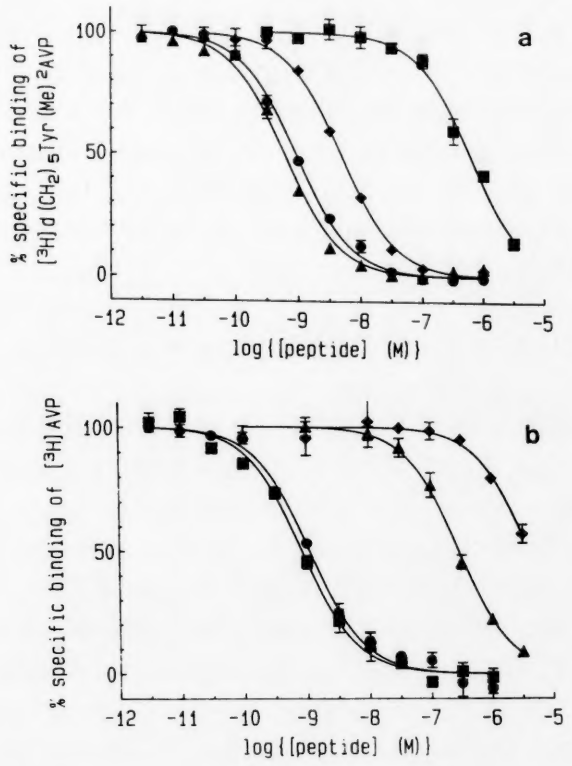

Fig. 2. Determination of dissociation constants of structural vasopressin analogues for the V_{1a} and V_2 vasopressin receptor subtypes. Membrane preparations from rat liver (*a*) and bovine kidney medulla (*b*) were incubated with 0.08–0.12 nM [^{3}H]d$(CH_2)_5$Tyr(Me)^{2}AVP and 0.3–0.6 nM [^{3}H]AVP respectively and various concentrations of the unlabelled peptides: (●) AVP; (■) dVDAVP; (▲) ALVP; (◆) ALBtnVP. Points are mean values ± SEM of triplicate determinations. Values for the specific binding of [^{3}H]tracer ligand in the presence of unlabelled peptide are expressed as a percentage of the specific binding in the absence of competing ligand. Curves represent the best fit of a theoretical simple Langmuir isotherm to the experimental data.

TABLE 1

BINDING AFFINITIES OF ALVP AND ALBtnVP AT THE V_{1a} AND V_2 VASOPRESSIN RECEPTOR SUBTYPES

The dissociation constants (K_d) of ALVP and ALBtnVP binding to the V_{1a} and V_2 receptor subtypes were determined by competition binding experiments as shown in Fig. 2. Data are mean values (±SEM) corrected for occupancy of [^{3}H]tracer ligand. Numbers in parentheses indicate the number of determinations for each value. The K_d values show that both ALVP and ALBtnVP are highly selective for the V_{1a} receptor subtype when compared with the natural agonist AVP.

Ligand	Binding affinity K_d (nM)		Selectivity V_{1a}/V_2
	V_{1a}	V_2	
AVP	0.68 ± 0.14 (5)	0.68 ± 0.09 (4)	1
ALVP	0.36 ± 0.05 (3)	194 ± 27 (6)	539
ALBtnVP	5.63 ± 0.44 (5)	2540 ± 500 (5)	451

0.19 ± 0.07 μmol/min/10^6 cells (mean ± SEM, $n = 5$) to 1.29 ± 0.19 μmol/min/10^6 cells ($n = 5$). ALVP completely blocked this AVP-stimulated glycogen phosphorylase *a* activity with a corrected IC_{50} of 2.6 nM. The antagonistic nature of the biotinylated analogue ALBtnVP was established by the observation that 1 μM ALBtnVP completely inhibited AVP-stimulated inositol lipid turnover in WRK-1 cells (data not shown).

Visualization of V_{1a} receptors on WRK-1 cells

Using TEM, WRK-1 cells with normal morphology were observed attached to the carbon coated colloidin film on electron microscope grids. When incubated with 20 nM ALBtnVP in concert with streptavidin-Au, gold particles were visible on the surface of all cells. Whilst individual gold particles were observed on the cell surface, clusters of gold particles, particularly on cell processes, were a consistent feature in these experiments (Fig. 3). Quantification of the number of gold particles/cell gave a value (mean ± SEM) of 671 ± 259 ($n = 4$). In parallel incubations cells were labelled with ALBtnVP in the presence of 10 μM

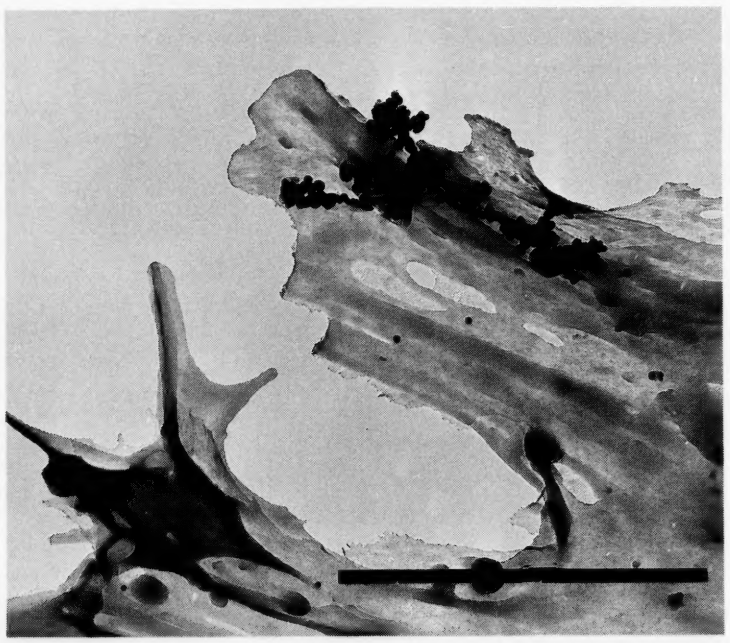

Fig. 3. Distribution of V_{1a} receptors on a WRK-1 cell revealed using ALBtnVP and streptavidin-Au. WRK-1 cells grown on colloidin coated electron microscope grids were morphologically similar to cells grown on glass or plastic surfaces. This cell was treated with ALBtnVP and streptavidin-Au and processed as described in Materials and Methods. Clusters of gold particles, particularly on cell processes, were a consistent finding on all similarly treated cells. ×38,300, bar = 1 μm.

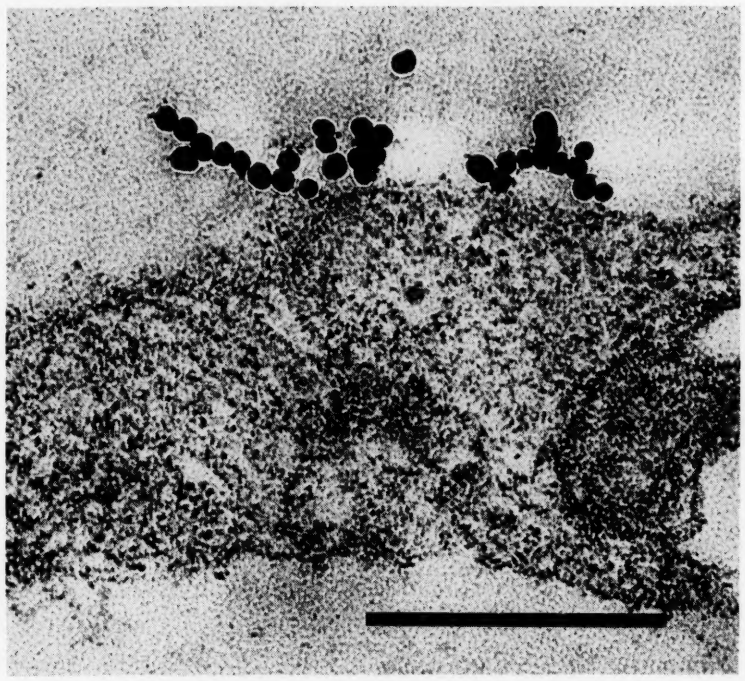

Fig. 4. Localization of the ALBtnVP/streptavidin-Au complex at the plasmalemma. Sections of WRK-1 cells, incubated with ALBtnVP and streptavidin-Au, were prepared as described in Materials and Methods. This figure demonstrates that gold particles were located exclusively on the external surface of the plasmalemma. No evidence for the internalization of receptor bound ALBtnVP/streptavidin-Au complex was observed in any preparation. ×122,300, bar = 0.25 μm.

of the V_{1a}-selective antagonist $d(CH_2)_5Tyr(Me)^2$-AVP. This reduced the number of gold particles observed to 11 ± 5/cell ($n = 4$) thereby demonstrating the receptor specific interaction of ALBtnVP with V_{1a} receptors. Control incubations in which the biotinylated ligand was omitted revealed that the non-specific binding of streptavidin-Au was negligible (mean number of gold particles 1.4 ± 0.6/cell, $n = 4$). Gold particles were observed on the external surface of the plasmalemma in some sections of similarly treated cells (Fig. 4). We did not observe any gold particles in sections prepared from WRK-1 cells treated with either ALBtnVP (20 nM) plus $d(CH_2)_5Tyr(Me)^2AVP$ (10 μM) or with streptavidin-Au alone. In all experiments we found no evidence for the internalization of streptavidin-Au as all gold particles were located exclusively on the external surface of the plasmalemma (Fig. 4).

Visualization of V_{1a} receptors in rat brain hippocampus

The use of formaldehyde (2.5% (w/v)) as a fixative together with glycergel as a mounting

medium allowed the specific fluorescence of fluorescein avidin DCS (yellow) to be observed above a low level of background autofluorescence (dull green). Using the incubation conditions described in Materials and Methods, ALBtnVP binding sites were observed with a punctate distribution on individual neurons located in the stratum radiatum of the CA1 and CA2 pyramidal cell layers of the dorsal hippocampus. Specific fluorescence was not improved by a longer incubation time with the biotinylated ligand or by increasing the concentration of fluorescein conjugated avidin. No specific fluorescence was observed in control experiments in which $d(CH_2)_5Tyr(Me)^2AVP$ (5 μM) was included during the incubation of brain slices with the biotinylated ligand. Moreover, when the specific oxytocin agonist Thr^4Gly^7OT (1 μM) was co-incubated with ALBtnVP the specific fluorescence was unchanged. This established that ALBtnVP was labelling AVP receptors and not oxytocin receptors.

Discussion

Biotinylated ligands which retain high affinity and pharmacological selectivity have proven to be useful tools for studying receptors (Hofmann et al., 1977; Redeuilh et al., 1985; Hazum et al., 1986; Rosenberg et al., 1986; Marie et al., 1990). The utility of such probes is based on the fact that labelled conjugates of avidin and streptavidin can be used to complex with high affinity to the biotin moiety of the ligand. Our aim was to synthesize a biotinylated probe for vasopressin receptors. In designing such a biotinylated ligand several factors were taken into consideration: (i) The ligand had to bind to vasopressin receptors with high affinity. (ii) As multiple subtypes of AVP receptor are expressed, pharmacological specificity was required so that a single isoreceptor (in this case V_{1a}) could be probed in a heterogeneous population. (iii) An antagonist was favoured, as this would ensure that the ligand's high affinity binding was insensitive to the degree of receptor : G-protein coupling. This precluded difficulties which can arise when interpreting differences in the level of agonist binding. (iv) Avidin and streptavidin labelled with various reporter groups must be able to bind with high affinity to the biotin moiety of the ligand. (v) To be useful, the ligand must be bi-functional in that it must be able to bind to V_{1a} receptors and to streptavidin/avidin conjugates simultaneously.

Kruszynski et al. (1980) have reported that the peptide $d(CH_2)_5Tyr(Me)^2AVP$ is a high affinity antagonist which is very selective for V_{1a} receptors. Consequently, this was selected as the ligand from which to develop a biotinylated probe. In an attempt to preserve the high affinity binding and marked V_{1a} selectivity of $d(CH_2)_5Tyr(Me)^2AVP$, it was judged necessary to perturb the conformation of $d(CH_2)_5Tyr(Me)^2AVP$ as little as possible whilst conjugating the biotin moiety. Previous investigations (Manning et al., 1984) have shown that the carboxy-terminal Gly or $GlyNH_2$ could be deleted or substituted with $AlaNH_2$, $SerNH_2$ or $ArgNH_2$ with little reduction in antivasopressor potency. This suggested that coupling biotin to the carboxy-terminus would be the least detrimental to the pharmacological characteristics which we were endeavouring to preserve. We synthesized the novel ligand ALVP which we have demonstrated in this paper to be similar to the parent peptide in that it is a high affinity antagonist with marked V_{1a} selectivity. However, unlike $d(CH_2)_5Tyr(Me)^2AVP$, ALVP is amenable to the coupling of functional groups onto the $LysNH_2$ ϵ-amino group via reaction with *N*-hydroxysuccinimide esters. Subsequently we synthesized the biotinylated ligand ALBtnVP. This molecule incorporated a long spacer-arm between the amino acid sequence of the ligand and the biotin moiety (Fig. 1) with the aim of reducing steric hindrance to avidin binding. This could be particularly important when avidin has to complex with the ligand when it is located in the binding pocket of a receptor protein. When biotin was conjugated to ALVP, the K_d increased 15-fold; however, with a $K_d = 5.63$ nM ALBtnVP is still a high affinity antagonist. Furthermore, ALBtnVP was demonstrated to be V_{1a} selective. Hence the rationale we had used to design the biotinylated probe had proved correct with respect to preserving the pharmacological characteristics of the precursor $d(CH_2)_5Tyr(Me)^2AVP$.

Experiments with WRK-1 cells and hippocampal slices demonstrated that ALBtnVP is bifunctional and is capable of binding avidin or

streptavidin whilst specifically labelling AVP receptors. V_{1a} receptors are readily detected on WRK-1 cells. However, there was no evidence for the internalization of the receptor : ligand complex. This is consistent with the findings of Cantau et al. (1988) who demonstrated that internalization of V_{1a} receptors via coated pits was stimulated by agonist occupancy of receptors, but was unaffected by antagonist binding. Previous studies (Brinton et al., 1984; De Kloet et al., 1985; Tribollet et al., 1988) have demonstrated that binding sites for [^{3}H]AVP can be demonstrated in neuroanatomically defined regions of the rat brain using ^{3}H autoradiography. Moreover, the hippocampus has been reported to express [^{3}H]AVP binding sites which display a pharmacological profile akin to that of the peripheral V_{1a} receptor (Barberis, 1983) and which couple to phosphinositidase C (Stephens and Logan, 1986). Although oxytocin receptors have been demonstrated in the hippocampus (Muhlethaler et al., 1983; Audigier and Barberis, 1985) co-incubation with excess of the selective oxytocin agonist Thr^{4}Gly^{7}OT failed to prevent ALBtnVP binding. This contrasts with the observations using excess of the V_{1a}-selective antagonist d$(CH_2)_5$Tyr(Me)^{2}AVP, thereby demonstrating that ALBtnVP selectively labelled V_{1a} vasopressin receptors on hippocampal neurons. The punctate distribution of fluorescent label on these neurons is suggestive of multiple vasopressinergic synapses.

Data presented here demonstrate that ALBtnVP should prove a useful probe for selectively localizing V_{1a} receptors in histological studies at the level of the light, or electron, microscope. In this regard, it is of note that while this paper was in preparation Jans et al. (1990) reported the synthesis of the biotinylated ligand des-[Dab-(biotinylamido)hexanyl^4,Arg^8]vasopressin which proved to be a non-selective agonist at AVP receptors. The different pharmacological characteristics of these two biotinylated probes should make their use complementary in future localization, and expression, studies.

Acknowledgements

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Antiestrogens stimulate expression of transiently transfected and endogenous genes in rat pituitary tumor cell lines

P. Reed Larsen and Robert Warne

Howard Hughes Medical Institute Laboratory and Thyroid Division, Department of Medicine, Brigham and Women's Hospital and Harvard Medical School, Boston, MA 02115, U.S.A.

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Summary

Tamoxifen, nafoxidine, and clomiphene (1×10^{-5} M) cause 5- to 15-fold increases in transient expression of plasmids transfected into rat somatomammotrophic pituitary tumor cell lines. To be effective, the antiestrogen must be present during the calcium phosphate transfection though it does not enhance the nuclear uptake or stability of transfected plasmid. The effect occurs with mammalian (rat growth hormone, mouse metallothionein I) or viral (thymidine kinase, Rous sarcoma virus) promoters and is inhibited by prior exposure of cells to high concentrations of estradiol but not glucocorticoid, progesterone or testosterone. *Cis*-tamoxifen, a conformation with much lower affinity for the estrogen receptor, has only one-fifth the effect of tamoxifen. Neither estradiol nor diethylstilbestrol have similar effects. Tamoxifen also increases endogenous rat growth hormone mRNA in these pituitary tumor cell lines. Transient expression in a number of other cell lines (JEG-3, COS-7, PC-12) is unaffected by tamoxifen suggesting the effect may be cell-type specific though MCF-7 cells are slightly responsive. The mechanism for the potent stimulation of gene transcription by these agents is not apparent but may be relevant to the mechanism of action of these agents as estrogen antagonists in vivo.

Introduction

Antiestrogens, such as tamoxifen, are assumed to act by binding to cellular estrogen receptors subsequently interfering with estrogen-induced transcription of hormone-dependent genes (Katzenellenbogen et al., 1979). The nature of the antiestrogen effect is not understood and other mechanisms, some only demonstrable at very high concentrations, have also been proposed. These include effects on calcium currents (Greenberg et al., 1987; Sartor et al., 1988), calmodulin antagonism (Lam, 1984; Fanidi et al., 1989), protein kinase C inhibition (O'Brian et al., 1985), and interactions with antiestrogen binding sites on cell membranes (Sudo et al., 1985; Thieulant et al., 1986). Dannies et al. (1977) and Amara and Dannies (1986) reported stimulation of prolactin and growth hormone synthesis in rat pituitary tumor cells by tamoxifen, clomiphene, and other antiestrogens. In subsequent studies, similar effects were demonstrated in primary cultures of rat

Address for correspondence: P.R. Larsen, Howard Hughes Medical Institute Laboratory and Thyroid Division, Department of Medicine, Brigham and Women's Hospital and Harvard Medical School, Boston, MA 02115, U.S.A.

pituitary cells using 10^{-7} M tamoxifen (Martinez-Campos et al., 1986). The present experiments were performed to explore the mechanism of the effect of antiestrogens using transient gene expression techniques.

Materials and methods

RNA isolation

Pituitary tumor cells (GH4C1, GC and GH3 cell lines) were grown in Ham's F10 medium/10% fetal bovine serum (FBS) and transfected in 60 mm tissue culture plates as previously described (Larsen et al., 1986). The GH4C1 cells were a gift from Dr. Armen Tashjian, Harvard Medical School. To prepare RNA, medium was removed and 1 ml of 4 M guanidinium thiocyanate solution added and the cells placed on ice. After sonication for 30 s, 0.5 ml 10 M lithium chloride was added and the mixture was incubated overnight at 4°C. Samples were microfuged for 10 min at 4°C and the pellets washed once with 2 mM lithium chloride/4 M urea at 4°C. After washing, pellets were resuspended in 400 μl of Tris (50 mM)-EDTA (1 mM) (TE), 1% sodium dodecyl sulfate (SDS) solution heated to 65°C and extracted immediately with phenol/isoamyl alcohol/chloroform (PIC). The PIC extraction was repeated a second time and then the RNA was precipitated with ethanol. The pellets were treated with DNase according to standard methods. After stopping the digestion, the samples were extracted with PIC followed by IC and then precipitated with ethanol.

Northern gels

RNA samples were resuspended at 2 μg/μl and 10 μg of each sample was run on a 1.1% agarose/formaldehyde gel according to standard methods (Ausubel et al., 1989). After electrophoresis the gel was placed in 10 × SSC (1 × SSC = 0.15 M NaCl-0.015 M Na citrate, pH 7.0) and gently shaken for 45 min. Gels were transferred to gene screen hybridization transfer membrane (New England Nuclear) in 20 × SSC buffer overnight and UV crosslinked to the membrane with a Stratalinker 1800 (Stratagene, La Jolla, CA, U.S.A.). The membrane was incubated at 42°C overnight in prehybridization buffer (25 mM potassium phosphate buffer pH 7.4, 5 × SSC, 5 × Denhardt's, 50 μg/ml salmon sperm DNA, 50% formamide). The blot was transferred to hybridization buffer containing 10% dextran sulfate and [^{32}P]cDNA probe added and incubated at 42°C overnight. Blots were washed in decreasing concentrations of SSC/0.1% SDS using standard techniques (Ausubel et al., 1989).

cDNA or cRNA probe preparation

An 800 bp rGH cDNA *Hin*dIII fragment was obtained from Dr. David Moore, at Department of Genetics, Harvard Medical School. This was labelled with the Prime Time 'C' method using the kit purchased from International Biotechnologies (New Haven, CT, U.S.A.) according to the directions of the manufacturer. A mouse β-actin cRNA probe was prepared from a mouse β-actin cDNA (Spiegelman et al., 1983) cloned into Bluescript (Stratagene, La Jolla, CA, U.S.A.) obtained from Dr. Joseph Majzoub (Children's Hospital, Boston, MA, U.S.A.). The plasmid was linearized with *Sma*I and transcribed with T3 polymerase according to the instructions of the manufacturer (Stratagene, La Jolla, CA, U.S.A.). A cRNA probe for the rGH237CAT was prepared from rGH1.8CAT described previously (Larsen et al., 1986) by the following method. A 1.3 kb *Pvu*II fragment was cut with *Bgl*II and ligated to a *Bam*HI/*Spe*I cut and filled in Bluescript KS vector (Stratagene). A cRNA probe was prepared from this by linearization with *Pst*I followed by proteinase K digestion and PIC and IC extraction. The cRNA was transcribed using T7 polymerase according to the instructions of the manufacturer. Analysis of rGH mRNA expression was performed using a laser densitometer (Molecular Dynamics) with reference to the expression of β-actin mRNA in the same lane.

Hirt plasmid DNA extraction and Southern blot

Transfected plates were washed once with phosphate-buffered saline (PBS) before addition of 0.2 ml of lysis buffer (10 mM EDTA, 0.6% SDS). The cell mixture was scraped off the plates and transferred to a 1.5 ml Eppendorf tube. The tubes sat at room temperature for 20 min with periodic vortexing and then 50 μl of 5 M NaCl were added and the tubes incubated overnight at 4°C. After microfuging for 3 min the supernates

were extracted twice with phenol. Liquid polyacrylamide (2 mg/ml) was added to a final concentration of 20 μg/ml and the solutions were ethanol precipitated. After centrifugation pellets were resuspended in 50 μl of TE. Each sample was digested with *Pvu*II prior to electrophoresis on a 1% agarose gel. After denaturation, the DNA was transferred to a Duralon UV filter (Stratagene) in 20 × SSC buffer (3 M NaCl, 0.3 M sodium citrate, pH 7.0) overnight. DNA was crosslinked to the filter using the Stratalinker 1800 and probed with the *Pvu*II cRNA fragment using standard techniques (Ausubel et al., 1989).

Transient expression assays of CAT and hGH

These were performed by phase extraction (Seed and Sheen, 1988) and immunometric assay (Nichols Institute, Los Angeles, CA, U.S.A.) as previously described (Larsen et al., 1986; Selden et al., 1986).

Results

Tamoxifen increases transient expression from both viral and eukaryotic chimeric promoter / reporter constructs in pituitary tumor cells

During initial studies in which antiestrogens were used to neutralize potential estrogen-like effects of phenol red on estrogen response element (ERE) containing plasmids, we evaluated the dose–response relationships of tamoxifen on transient gene expression. We noted that tamoxifen caused marked increases in expression of human growth hormone (hGH) in rat pituitary tumor cell lines transfected with the mouse metallothionein I-hGH transfection efficiency control plasmid, pXGH5. Stimulation of expression from the rat GH-chloramphenicol acetyl transferase vector, rGH237CAT also occurred. This effect was detectable at 3×10^{-6} M tamoxifen and peaked at 10^{-5} M (Fig. 1). Tamoxifen citrate and the free acid were equally effective. At concentrations of 3×10^{-5} M tamoxifen, significant cell toxicity appeared with decreases in hGH and CAT expression. These effects were observed in GH4C1, GC, and GH3 pituitary tumor cell lines and occurred with all promoters used including mouse metallothionein I, Rous sarcoma virus LTR (RSV), thymidine kinase (TK), and rGH (Fig. 2). Both

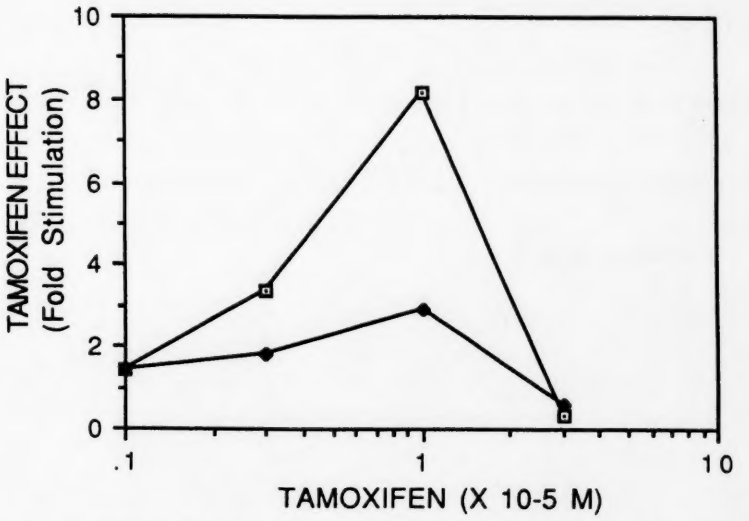

Fig. 1. Effect of tamoxifen on transient expression of human growth hormone directed by mouse metallothionein I promoter (pXGH5) and CAT directed by the rGH promoter (rGH237CAT). GH4C1 pituitary tumor cells were transfected with pXGH5 (5 μg) and rGH237CAT (10 μg) using calcium phosphate with or without tamoxifen. Control plates received vehicle (ethanol) alone. Results are plotted as the ratio of hGH (ng/ml media, open symbols) or CAT (percent butyrylated CAT/100 μl cell extract, solid symbols) to that present in a paired untreated control plate. Results shown are typical of ten similar experiments with each dose level examined in duplicate. The range of stimulation in GH4C1 cells was 7- to 20-fold for hGH and 3- to 9-fold for CAT.

hGH and CAT reporter expression were stimulated to roughly comparable degrees (Fig. 2). The transient transfections of these cells were per-

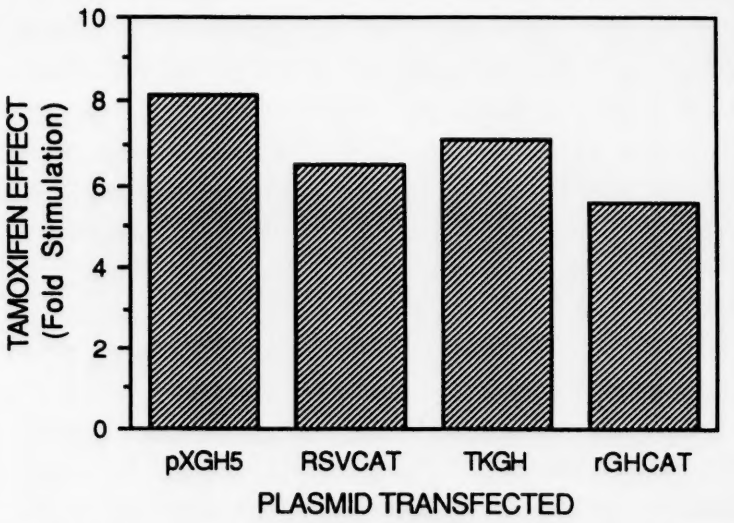

Fig. 2. Effect of tamoxifen on transient expression by different promoters. Indicated plasmids were transfected into GC pituitary tumor cells in pairs using calcium phosphate coprecipitation. Tamoxifen (1×10^{-5} M) was present the day of transfection and cells and/or media were harvested 48 h after transfection. Medium hGH or CAT in the cell extract was compared to that in paired plates exposed only to vehicle.

TABLE 1

EFFECT OF THE TIMING OF TAMOXIFEN EXPOSURE ON THE TRANSIENT EXPRESSION OF hGH AND CAT BY GH4C1 CELLS

$CaHPO_4$-mediated transfection with pXGH5 and rGH237CAT was performed as described over 0–24 h. Cells and media were harvested at 48 h.

Time of exposure (h)	hGH tamoxifen/control	CAT tamoxifen/control
0–24	10.1	13.2
24–48	1.4	1.1
0–48	17.8	11.0

formed by calcium-phosphate-DNA coprecipitation and we noted that tamoxifen had to be present during the 24 h transfection process to be effective (Table 1). The stimulation was already about 50% of maximum at the end of the transfection at 24 h.

The increase in transient expression parallels antiestrogen potency

Stimulation of expression was also observed with other antiestrogens including clomiphene and nafoxidine as well as with 4-hydroxy-tamoxifen (Fig. 3). Dose–response curves showed that the effect of these agents was also maximum at 10^{-5} M. To determine whether these effects required a potent antiestrogen structure, we obtained a sample of the *cis* isomer of tamoxifen, ICI 47,699, from ICI Pharmaceuticals Group, Macclesfield, U.K. This compound has an affinity for the estrogen receptor less than 10% that of the *trans* isomer (Sutherland, 1981; Sudo et al., 1983). At 10^{-5} M, a maximally effective dose, *cis*-tamoxifen was only 20% as effective as the *trans* isomer in inducing transient expression. Higher concentrations (3×10^{-5} M) produced cell death as with the *trans* isomer.

We also explored the capacity of several hormonal steroids to inhibit the effects of these antiestrogens on transient expression. At concentrations of 10^{-6} to 10^{-5} M, only estradiol inhibited the stimulation by tamoxifen (Fig. 4). At lower concentrations, estradiol was ineffective. None of these hormones affected transient expression at 10^{-6} to 10^{-5} M nor did diethylstilbestrol (10^{-6} to 10^{-5} M). Since it has been speculated

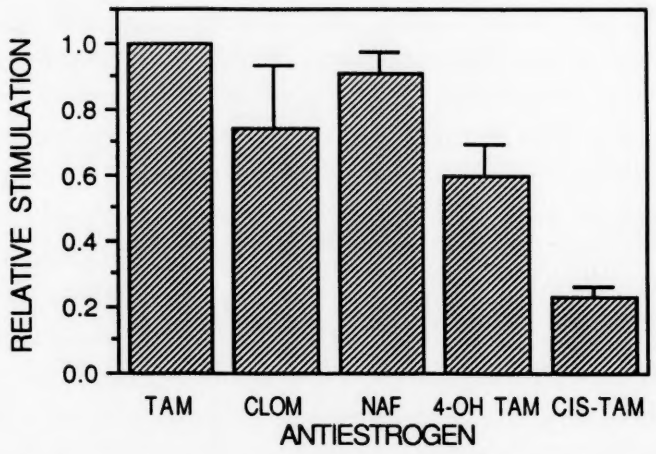

Fig. 3. Comparison of the effect of different antiestrogens on the transient expression of pXGH5 in pituitary tumor cells. Shown are the mean results in 2–3 experiments for each compound. Results are expressed relative to the effect of tamoxifen (1×10^{-5} M) as an internal control in each experiment. The mean response to tamoxifen was 9.5 ± 2.2 (SE)-fold. Clomiphene (CLOM), nafoxidine (NAF), 4-hydroxy-tamoxifen (4-OH TAM), and *cis*-tamoxifen (CIS-TAM) were all present during the 24 h transfection at 1×10^{-5} M. Expression in treated plates was compared with that in paired plates receiving only vehicle. At least three pairs of plates were used for each compound. Dose–response curves showed that 1×10^{-5} M gave maximal effects for each compound.

that calmodulin antagonism may be an important effect of tamoxifen at high concentrations (Lam, 1984; Fanidi et al., 1989), we also examined the

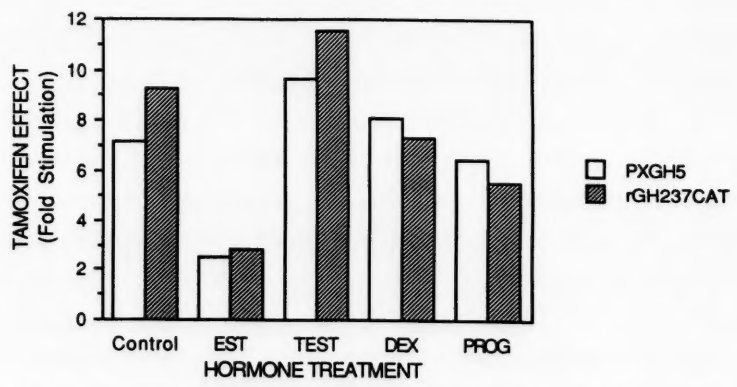

Fig. 4. Effects of pre-exposure of pituitary cells to various steroid hormones on the subsequent effect of tamoxifen. Pituitary tumor cells (GC) were transferred to medium containing 10% charcoal-stripped fetal bovine serum (FBS) containing the indicated hormones at zero time. Serum contained 17β-estradiol (EST), testosterone (TEST), dexamethasone (DEX), or progesterone (PROG) respectively at 1×10^{-5} M. After 24 h, cells were transferred with pXGH5 and rGH237CAT in the presence or absence of 1×10^{-5} M tamoxifen. Medium was changed 24 h later with continued hormone treatment. Cells and media were harvested after a further 24 h. Results presented are the ratios of expression of hGH or CAT in tamoxifen- to that in vehicle-treated plates.

TABLE 2

EFFECT OF TAMOXIFEN (1×10^{-5} M) ON pXGH5-INDUCED TRANSIENT hGH EXPRESSION IN SEVERAL TUMOR CELL LINES

$CaHPO_4$-mediated transfection of pXGH5 was performed from 0 to 24 h. Tamoxifen was present from 0 to 48 at which time the medium was harvested for hGH. Results are the mean ± SE of at least six paired plates.

Cell type	Relative hGH expression tamoxifen/control (mean ± SE)
GC	10.7 ± 1.5
GH4C1	9.0 ± 2.0
GH3	5.5 ± 0.5
AtT20	3.1 ± 0.2
MCF-7	2.1 ± 0.3

effects of calmidazolium and W-7 (*N*-(6-aminohexyl)-*S*-chloro-1-naphthalenesulfonamide hydrochloride) on transient expression in pituitary tumor cell lines. Neither of these agents had any detectable effect at concentrations ranging from 10^{-8} to 10^{-5} M (data not shown) and concentrations greater than 10^{-5} M were toxic.

The stimulation of transient expression is dependent on cell type

Tamoxifen caused 5- to 12-fold increases in transient expression in GC, GH4C1, and GH3 somatotroph/mammotroph cell lines. The stimulation of expression in AtT20 and MCF-7 cells was much lower but detectable (Table 2). No effects of nafoxidine or tamoxifen were observed in several other tumor cell lines including neuroblastoma (NB41A3), PC-12 (adrenal medulla), COS-7, CV-1 (both monkey kidney) or JEG-3 (choriocarcinoma).

Tamoxifen does not increase transfection efficiency

Since to be effective, the antiestrogens had to be present during the calcium phosphate transfection (Table 1), it seemed possible that these agents might be facilitating the nuclear uptake or retention of the transfected DNA. Accordingly, we studied the GH4C1 cell content of transfected plasmid rGH237CAT. The results of a Southern analysis of a plasmid DNA extract from three pairs of plates probed with an rGH237CAT cRNA are shown in Fig. 5. There was no increase in the cell content of transfected plasmid induced by tamoxifen even though, in the same cells, hGH expression directed by pXGH5 was induced 4.5-fold.

Tamoxifen stimulates endogenous rGH gene expression in GH4C1 cells

Since 4-fold increases in prolactin have been reported during 1 week exposure of GH4C1 cells to 10^{-6} M tamoxifen (Amara and Dannies, 1986), we examined its effects on rGH mRNA expression. Three- to 5-fold increases in cytoplasmic rGH mRNA (as well as a larger apparent nuclear precursor) were observed within 24 h (Fig. 6). An internal control for generalized mRNA synthesis was obtained by probing these blots with a mouse β-actin cRNA. The 800 nucleotide cytoplasmic rGH mRNA was increased 5.6-fold relative to that for β-actin. The cytoplasmic level of the latter mRNA was not significantly altered by 10^{-5} M tamoxifen nor was total cellular protein increased during tamoxifen exposure. Three- to 4-fold stimulation of rGH expression by tamoxifen was

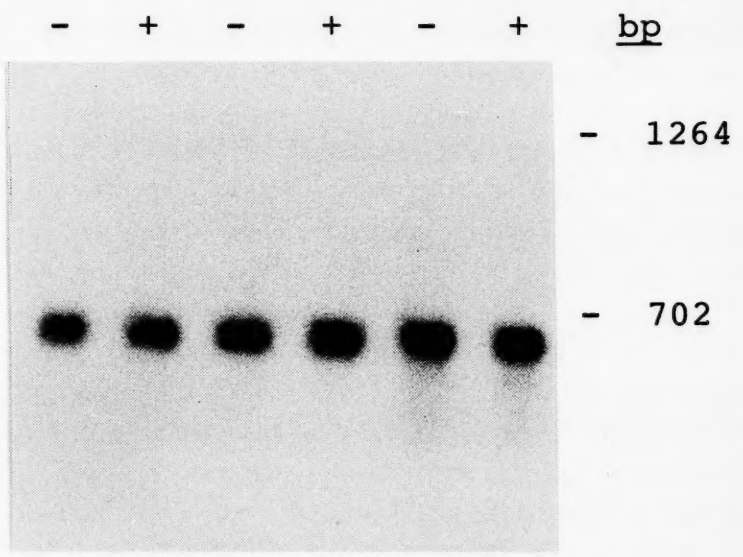

Fig. 5. Southern blot of a Hirt extract of GH4C1 cells which had been transfected 24 h earlier with rGH237CAT with or without tamoxifen (1×10^{-5} M). The extracted plasmid DNA was digested with *Pvu*II which should release an approximately 650 bp fragment from rGH237CAT. This fragment includes the 237 bp rGH promoter region and 400 bp of the adjoining vector sequences. The blot was probed with a rGH237CAT cRNA prepared as described. The autoradiograph was exposed for 15 min. 10 μg of DNA were present per lane and the minus and plus signs above each lane refer to replicate samples exposed to either 1×10^{-5} M tamoxifen (+) or vehicle (−).

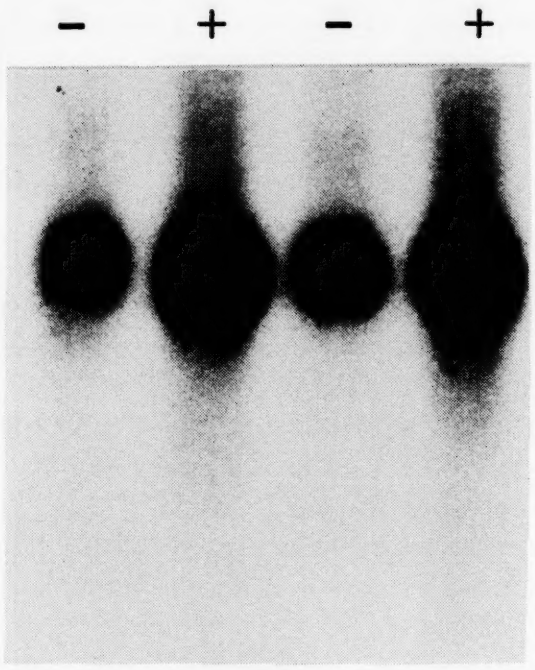

Fig. 6. Northern blot of GH4C1 cells exposed (+) or not exposed (−) to 1×10^{-5} M tamoxifen for 24 h. 10 μg of total cellular RNA were applied per lane and hybridized to an 800 bp rGH cDNA probe.

observed in an identical experiment using GC cells.

Tamoxifen attenuates the T_3-dependent expression induced by a mutant of the rGH promoter

Our previous studies have demonstrated that there is an inverse relationship between the basal expression of a triiodothyronine (T_3) response element (T_3RE) linked promoter in the absence of T_3 and its expression in the presence of the ligand (Brent et al., 1989a, b, c). To show that cells maintained physiological responsiveness to other transcriptionally active agents after exposure to these high levels of antiestrogens, we examined basal and T_3-induced CAT expression using a wild-type and a highly T_3-responsive rGH promoter mutant, GB35 (Brent et al., 1989b). Tamoxifen increased expression of both hGH, the internal control reporter, and CAT, the T_3-dependent reporter, although the internal control was increased to a greater extent than was CAT (Table 3). The net T_3 induction of rGH237CAT was modestly reduced from 2.7- to 1.9-fold. The plasmid GB35 was 3- to 4-fold more responsive to T_3 than was the wild-type promoter by virtue of both a decrease in the basal expression (no T_3 present) as well as an increase in expression in the presence of

TABLE 3

EFFECTS OF INCREASING TRANSIENT EXPRESSION WITH TAMOXIFEN ON THE SUBSEQUENT RESPONSE OF T_3RE-rGH PROMOTER-CAT CONSTRUCTS TO T_3

Cells were pre-incubated in 10% charcoal-stripped (T_3-free) FBS containing 10^{-7} M dexamethasone for 24 h. They were then transfected with plasmid DNA in the presence or absence (control) of 10^{-5} M tamoxifen by a 24 h exposure to $CaHPO_4$/DNA precipitates (Larsen et al., 1986). At this time, the medium was changed with half the paired plates from each group receiving medium containing 10% charcoal-stripped FBS $+5\times10^{-8}$ M T_3. Tamoxifen was not present during the second 24 h incubation. The medium was sampled for hGH concentration and cell lysates prepared for CAT assay after 24 h exposure to conditions with or without T_3.

Plasmid	T3	Control mean CAT/hGH [a]	Tamoxifen (10^{-5} M) mean CAT/hGH [a]
rGH237CAT	0	0.29	0.14 [b]
	5×10^{-8} M	0.81	0.26
	T_3 effect	2.7	1.9
GB35 [c]	0	0.23	0.11
	5×10^{-8} M	2.20	0.47
	T_3 effect	9.5	4.3

[a] Percent chloramphenicol butyrylated/100 μl cell extract/18 h ÷ (cpm ^{125}I-hGH)$^{-3}$/100 μl medium. Results are the mean of duplicate pairs of plates in a typical experiment.

[b] Tamoxifen caused an approximately 8-fold increase in hGH expression from pXGH5 and a 3- to 4-fold increase in CAT expression.

[c] GB35 is a 137 nucleotide rGH promoter CAT construct with an oligonucleotide containing an up-mutation of the T3 response element inserted upstream (Brent et al., 1989b).

T_3 (Brent et al., 1989b). In the tamoxifen-pretreated cells, the increment in CAT expression in the presence of T_3 was lower relative to basal CAT expression than it was in the absence of this agent. Thus, T_3 induction of CAT expression was preserved after tamoxifen exposure though at a reduced level.

Discussion

The effects of the antiestrogens on transient expression are similar to those described earlier for regulation of GH and prolactin in pituitary tumor cells and in primary cultures of rat pituitaries (Dannies et al., 1977; Amara and Dannies,

1986; Martinez-Campos et al., 1986). However, in those studies, the antiestrogens were present at lower concentrations, required one to several weeks for maximal effects and the magnitude of the response was less (2- to 4-fold). Similar to the present effects, however, they were inhibited by estrogen and required an antiestrogen conformation with a high affinity for the estrogen receptor. The *trans* isomer of tamoxifen was 5-fold more effective than *cis*-tamoxifen, which has a much lower affinity for the estrogen receptor consistent with estrogen receptor effects, though unrecognized differences in the cell uptake or metabolism of *cis*-tamoxifen could also explain these results (Sutherland, 1981). Further evidence that estrogen receptor is involved in this effect is its attenuation by estradiol but not by exposure to similar levels of dexamethasone, testosterone, or progesterone. On the other hand, if estrogen receptors are involved such receptors do not have typical affinities for either ligand or antiestrogen. The concentrations of both agents required for these effects are 1 to 2 orders of magnitude higher than are required for most estrogen receptor-dependent effects. In fact, maximum effective antiestrogen concentrations (10^{-5} M) are only 3-fold below toxic levels (Fig. 1). Nonetheless, the fact that the striking effects demonstrated are positive and that cells pretreated with tamoxifen respond appropriately to another ligand-dependent transcriptional stimulator, T_3, indicates that they remain biochemically intact. A variety of potential mechanisms involving other actions of antiestrogens such as calmodulin antagonism or phosphodiesterase inhibition as well as binding to incompletely characterized antiestrogen binding sites (Sudo et al., 1983; Thieulant et al., 1986) seem to be less likely on the basis of the stereospecificity of the tamoxifen effect and the absence of stimulation by calmidazolium or W-7.

The mechanism for the tamoxifen-induced increase in expression of transiently transfected promoters appears to be increased transcription. Although the antiestrogens need only be present during the transfection period, there is no evidence for an increase in the amount of transfected nuclear DNA in tamoxifen-treated cells (Fig. 5). The presence of stimulation within the first 24 h and the lack of tamoxifen effect during the second day after transfection argue against mRNA stabilization and enhanced translation as a mechanism since 24–48 h is the time of peak expression in this system (Larsen et al., 1986). The effect is not promoter-specific occurring with both viral and eukaryotic promoters. This suggests a generalized stimulation of transcription by antiestrogens which is consistent with the effect on the endogenous rGH gene (Fig. 6). However, an increase in β-actin mRNA does not occur and there was, in addition, no effect on total cellular protein in cultures exposed to tamoxifen for 24–48 h. Thus there may be different mechanisms for the effects to antiestrogens on transfected and endogenous genes. Neither estradiol nor diethylstilbestrol (10^{-5} M) stimulate transient expression suggesting that this is peculiar to antiestrogens.

As mentioned, it is notable that despite 5- to 15-fold increases in transient expression by tamoxifen, the cells maintain the capacity to respond to another transcriptionally active hormone, T_3. The modest attenuation of the T_3 stimulation of rGH promoter-directed CAT expression (Table 3) could be a function either of a specific estrogen-like effect of tamoxifen to increase basal rGH promoter activity or a limitation of the T_3-dependent stimulation due to exhaustion of required nuclear transcription factors.

The antiestrogens are most effective in rat pituitary tumor cell lines although there was much lower, but still significant, stimulation of transient expression in the AtT20 ACTH-producing mouse pituitary tumor lines and MCF-7 human breast carcinoma cells. It is not known whether AtT20 cells express estrogen receptors. Of interest is the fact that the rat somatomammotropic tumor lines, the most responsive of all cell lines tested, were originally induced by either pituitary irradiation or long-term exposure of rats to diethylstilbestrol (Brent et al., 1989a, b, c). In both cases, hyperestrogenism, endogenous or exogenous, is thought to play a major role in the early phase of tumorigenesis (Brent et al., 1989b). Cells with abnormal, perhaps even constitutively active, estrogen receptors, could be selected for by such techniques. Inactivation of such receptors by antiestrogens could lead to a burst of transcriptional activity. The rapid and specific effect of antiestrogens on gene expression demonstrated in these cells sug-

gests their suitability as a model to explore the as yet incompletely understood actions of these important compounds.

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Synthesis and processing of pro-ocytocin in bovine corpus luteum and granulosa cells

Maryse Camier, David Benveniste, Nicole Barré, Noureddine Brakch and Paul Cohen

Groupe de Neurobiochimie Cellulaire et Moléculaire, URA 554 CNRS, Université P. et M. Curie, Paris, France

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Key words: Pro-ocytocin convertase; Neurophysin; Progesterone; Follitropin; Western blot

Summary

Bovine corpus luteum is the site of intense production of pro-ocytocin-neurophysin mRNA at day 1 after estrus (Ivell et al. (1985) FEBS Lett. 190, 263–267) which is followed by apparent delayed production of ocytocin. Therefore it is a good model to study both the translational and post-translational production of this neuropeptide in non-hypothalamic tissues and its regulation. In order to assess if this mRNA is translated during the lag period we have analyzed the neurophysin-like species produced in this organ. As early as day 2 after estrus one neurophysin species (pI ~ 4.7) could be detected and was unequivocally identified as pro-ocytocin-neurophysin. In primary cultures of luteinizing granulosa cells, biosynthetic intermediates were characterized, i.e. ocytocin-Gly, ocytocin-Gly-Lys and ocytocin-Gly-Lys-Arg, whereas amidated, fully mature, ocytocin was undetectable. We conclude that translation of pro-ocytocin-neurophysin mRNA takes place soon after transcription and we propose that incomplete processing could be responsible for the low level of ocytocin in the early bovine corpus luteum.

Introduction

Ocytocin is secreted by the ovary (Flint and Sheldrick, 1982; Whates and Swann, 1982) and is synthesized, via a common precursor to neurophysin, i.e. pro-ocytocin-neurophysin, by the corpus luteum (Rodgers et al., 1983; Swann et al., 1984). The essential features of this biosynthesis are similar in both corpus luteum and hypothalamus (Ivell and Richter, 1984; Swann et al., 1984). The same dibasic selective endoprotease, putative processing enzyme of pro-ocytocin-neurophysin which cleaves synthetic substrates at the restriction sequence (Gly-Lys-Arg) between ocytocin and neurophysin I, was found in both the hypothalamo–neurohypophyseal axis and corpus luteum (Clamagirand et al., 1986, 1987a; Créminon, 1989; Plevrakis et al., 1989). Together with the identification of COOH-terminally extended forms of ocytocin (ocytocin-Gly-Lys-Arg, ocytocin-Gly-Lys and ocytocin-Gly) in extracts of corpus luteum these observations argue strongly in favor of a comparable processing scheme (Clamagirand et al., 1987a).

In the follicles, the concentration of mature ocytocin appears to be rather low (Schams et al., 1985) as well as is the level of pro-ocytocin-neurophysin mRNA (Ivell et al., 1985). According to Ivell et al. (1985) ovulation triggers the transcrip-

Address for correspondence: Prof. P. Cohen, Groupe de Neurobiochimie Cellulaire et Moléculaire, URA 554 CNRS, Université P. et M. Curie, 96 Boulevard Raspail, 75006 Paris, France.

tion of the pro-ocytocin-neurophysin gene since pro-ocytocin-neurophysin mRNA concentration is increased 1 day after estrus, reaches a maximum in about 3 days, then decreases drastically. In contrast, the transcriptional expression of the pro-ocytocin-neurophysin gene is not accompanied by the appearance of measurable ocytocin immunoreactivity, since 4–5 days elapsed before significant amounts of this compound could be detected (Fehr et al., 1987). This immunoreactive ocytocin peaks 8–13 days after estrus then decreases dramatically. This apparent gap between the transcription of pro-ocytocin-neurophysin gene and appearance of immunoreactive ocytocin could be explained in essentially two ways: (i) no translation of pro-ocytocin-neurophysin mRNA occurs at first, (ii) translation of pro-ocytocin-neurophysin mRNA operates, but processing of the prohormone is absent or incomplete.

In the present paper we report on results obtained both in vivo and in vitro on bovine corpus luteum and on cultured granulosa cells which strongly support the conclusion that early translation of pro-ocytocin-neurophysin mRNA into pro-ocytocin-neurophysin occurs and is followed by the rate-limiting formation of incompletely processed forms of ocytocin, i.e. ocytocin-Gly-Lys-Arg, ocytocin-Gly-Lys, ocytocin-Gly.

Materials and methods

Neurophysin isolation

Cows, superovulated and bred as previously described (Clamagirand et al., 1987a) were used as a source of corpus luteum. Animals were sacrificed at different stages after estrus and ovaries were immediately collected and transported onto ice to the laboratory. Neurophysins, small proteins of $M_r \sim 10{,}000$, were extracted from corpora lutea as usual (Camier et al., 1973), in the presence of protease inhibitors (4 μg/ml of aprotinin, 1 μg/ml of pepstatin and 5 mM phenylmethylsulfonyl fluoride), and obtained hormone-free with others proteins of similar M_r by two successive molecular sieve filtrations.

Neurophysin characterization

Proteins ($M_r \sim 10{,}000$) were analyzed by isoelectric focusing (IEF) using the Phast System apparatus (Pharmacia, Uppsala, Sweden) with Phast Gel IEF 4–6.5 and a separation method recommended by the manufacturer. Then the proteins were transferred by diffusion to nitrocellulose membrane (Hybond C, Amersham, U.K.) at 4°C for 90 min in the presence of 25 mM Tris, 150 mM glycine, pH 8.3. After transfer the membrane was treated with 3% bovine globulin-free albumin (Sigma, St. Louis, MO, U.S.A.) in 10 mM phosphate buffer pH 7.5 containing 130 mM NaCl. Then it was incubated with rabbit anti-bovine neurophysin I serum (618-11) prepared in the laboratory. BioRad's goat anti-rabbit IgG alkaline phosphatase conjugate and color development reagents, 5-bromo-4-chloro-3-indolyl-phosphate *p*-toluidine salt and nitroblue tetrazolium chloride, were used to visualize neurophysin-anti-neurophysin complexes (BioRad, Richmond, CA, U.S.A.).

In order to characterize pro-ocytocin-neurophysin, proteins $M_r \sim 10{,}000$ prepared from corpora lutea at 2 or 3 days after estrus were submitted to hydrolytic cleavage by trypsin and by pro-ocytocin convertase. 1 μg of *N*-tosyl-L-phenylalanine chloromethyl ketone (TPCK)-treated trypsin (Sigma, St. Louis, MO, U.S.A.) was incubated with proteins, freed of protease inhibitors by previous filtration on Sephadex G-25 and containing 2 μg of immunoreactive neurophysin species, in 100 mM ammonium bicarbonate pH 8 at 37°C for 60 min. Reaction was stopped by addition of 0.1 N HCl. Immunoreactive ocytocin was measured by radioimmunoassay (RIA) and the released peptides were identified by high-performance liquid chromatography (HPLC). Pro-ocytocin convertase was purified by isoelectric focusing as previously described (Clamagirand et al., 1987b). Proteins $M_r \sim 10{,}000$ containing 9 μg of immunoreactive neurophysin were incubated with the convertase in 100 mM ammonium acetate pH 7 at 37°C for 16 h. After acidification, the reaction mixture was analyzed by HPLC.

Granulosa cell culture

Cows, superovulated and bred as previously described (Clamagirand et al., 1987a), were used as a source of granulosa cells. Animals were sacrificed 40 h after the beginning of the estrus. Ovaries were immediately collected, washed with

sterile 0.9% NaCl and immerged in cold culture medium before transportation to the laboratory. Non-ovulated follicles were used. At first, follicular fluid was removed by aspiration with a 27.5 gauge needle and 5 ml syringe and replaced by culture medium. Then granulosa cells were harvested through a slit by gentle scraping of the inner wall using a micro-inoculation loop. Medium and cells were recovered with a Pasteur pipette and centrifuged ($150 \times g$, 5 min). Cell pellet was washed with culture medium then the cells were resuspended in culture medium, counted and distributed into Nunclon Delta 24-well plates (Nunc, Copenhagen, Denmark): 0.3 to 1×10^6 cells in 1 ml of culture medium/well. The culture medium was Dulbecco's modified Eagle's medium and Ham's mixture F-12 (DME/F-12) containing 100 U/ml of penicillin and 0.1 mg/ml of streptomycin (Eurobio, Paris, France), 2 μg/ml of insulin and without fetal calf serum.

The cells were incubated at 37°C in a 5% CO_2 humidified atmosphere (Sanyo Incubator, Tokyo, Japan). Two to 4 hours after incubation, attached cells were washed with culture medium in order to eliminate dead cells and red blood cells. Then cultures were carried out in the presence or absence of the following hormones: follitropin (FSH) 1.5 mU (NIH-FSH-S1)/1 ml of culture medium, 0.5 μM testosterone or dehydrotestosterone. Culture media were changed daily. Cell number was deduced from DNA concentration as indicated by Jungclas and Luck (1986). DME/F-12 and hormones were supplied by Sigma (St. Louis, MO, U.S.A.).

Neurophysin and ocytocin isolation from culture media

Before RIA, immunoreactive neurophysin and ocytocin secreted into the culture media were isolated using Sep-Pak C-18 cartridges (Waters Assoc., San Francisco, CA, U.S.A.). 1 ml of culture medium was filtered through the cartridge previously washed with methanol, water and 0.1% trifluoroacetic acid, and elution was obtained with 3 ml of 40% acetonitrile in 0.1% trifluoroacetic acid. Eluate was evaporated in a Speed Vac Concentrator (Savant Instrument Co., Hicksville, NY, U.S.A.) then dissolved into RIA buffer.

HPLC identification of ocytocin peptides was also performed on culture media purified using Sep-Pak C-18 cartridges. The procedure was the same but elution was obtained with 20% acetonitrile in 0.1% trifluoroacetic acid. The recovery yield of ocytocin-Gly-Lys-Arg, ocytocin-Gly-Lys, ocytocin-Gly and ocytocin-NH_2 was determined as 83%, 77%, 67% and 69%, respectively.

Neurophysin, ocytocin and progesterone RIA

Neurophysin RIA was carried out with bovine neurophysin I purified (Camier et al., 1973) as standard and rabbit anti-neurophysin I serum (618-12) prepared in the laboratory and used at a final dilution of 1/75,000. Separation of free from bound neurophysin was obtained by zirconyl phosphate (Robinson et al., 1971). Sensitivity was 40 pg and 50% displacement of the [^{125}I]neurophysin I was obtained by either 0.6 ng of neurophysin I or 200 ng of neurophysin II.

Ocytocin RIA was carried out as previously described (Clamagirand et al., 1987a). Ocytocin and C-terminally extended ocytocin-peptides used as reference in RIA or HPLC were synthesized in the laboratory (Creminon et al., 1988). A minimum of 2 pg of ocytocin was detected and 50% displacement of the tracer ([^{125}I]ocytocin) was obtained with 75 pg of ocytocin, 300 pg of ocytocin-Gly-Lys-Arg, 250 pg of ocytocin-Gly-Lys and 210 pg of ocytocin-Gly, thus allowing a sensitive detection of the mature ocytocin and the corresponding extended forms.

Concentrations of progesterone in culture media were directly determined using a RIA kit (PROG-CT) (CIS, Bio Industries, France). Sensitivity was 15 pg.

Results

It is clear from previous work that pro-ocytocin-neurophysin proteolytic processing can be monitored by an evaluation of neurophysin and ocytocin production, or of the COOH-terminally extended forms of ocytocin (Clamagirand et al., 1987a; Brakch et al., 1989). Therefore, in order to assess translational expression of pro-ocytocin-neurophysin mRNA, the presence of mature neurophysin or of neurophysin-like material was determined in the early bovine corpus luteum after

TABLE 1

CONCENTRATIONS OF IMMUNOREACTIVE NEUROPHYSIN IN BOVINE CORPORA LUTEA

At different stages after estrus, corpora lutea were collected from cows superovulated and bred. Neurophysins were extracted by 0.1 N HCl then isolated by NaCl precipitation followed by Sephadex G-25 and G-75 filtrations. Protease inhibitors were used at all steps. Neurophysin concentrations correspond to the immunoreactivity measured by RIA at the elution volume of the proteins $M_r \sim 10{,}000$ on Sephadex G-75 and expressed as bovine neurophysin I. The values shown represent a mean evaluation made on several corpora lutea collected from 1–7 animals.

Days after estrus	Number of animals	Neurophysin (μg/g fresh tissue)
2	2	9
3	1	21
4–5	2+2	27
7–8	4+3	41
14	1	1.7

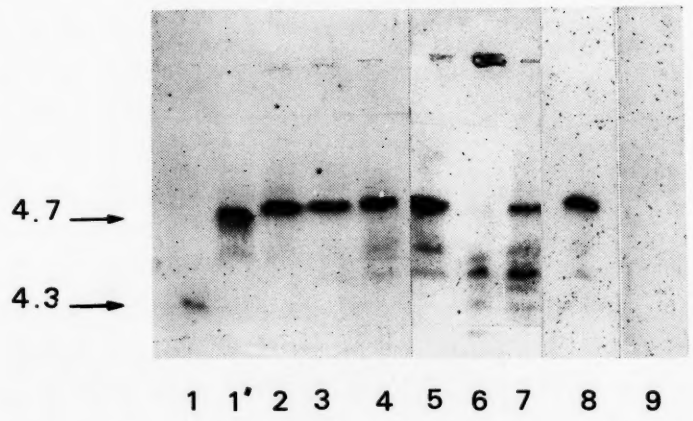

Fig. 1. Western blotting of proteins $M_r \sim 10{,}000$ isolated by Sephadex G-75 filtration and separated by IEF on a pH 4–6.5 gradient gel. Lanes 1 and 1′: standard bovine neurophysin I and II respectively. Lane 9: mixture of the standards non-incubated with antineurophysin I. Lanes 2–6: neurophysin of corpora lutea at 2, 3, 4–5, 7–8, 14 days after estrus. Lane 7: neurophysin of corpora lutea collected from cows slaughtered at an abattoir. Lane 8: neurophysin secreted by granulosa cells at 4, 5 and 6 days of culture. Arrows indicate the apparent p*I* of the bovine neurophysins I and II: 4.3 and 4.7, respectively.

ovulation. Table 1 gives the quantities of neurophysin measured by RIA in bovine corpora lutea taken at various stages. Neurophysin immunoreactivity is detected as early as day 2 after estrus then increases during development of the corpus luteum. The highest content is at days 7 and 8 after estrus. It is noteworthy that neurophysin immunoreactivity at days 2 and 3 is very high (25 and 50% of the content at days 7 and 8, respectively). These results obviously concern a period preceding the phase of maximal production of ocytocin as reported (Schams et al., 1985), i.e. from days 8–13. In an old corpus luteum of 14 days a low immunoreactivity is detected, in agreement with previous reports (Schams et al., 1985; Fehr et al., 1987) that ocytocin disappears at that stage. From days 2–8, immunoreactivity is noticeably higher than previously reported by others (Wathes et al., 1984) and this observation is not related to hormonal treatment since untreated abattoir animals slaughtered at luteal stage II according to Ireland et al. (1980) give similar data (about 58 μg of neurophysin/g wet tissue).

In order to identify the immunoreactive material, this was submitted to isoelectric focusing. In the corpus luteum, extracted neurophysins are largely contaminated by other protein species in contrast to the neurohypophysis where neurophysins represent almost the totality of $M_r \sim 10{,}000$ protein material. Therefore, after separation, the neurophysin species were identified by Western blot using anti-bovine neurophysin I antibodies. Fig. 1 shows that no neurophysin I, the ocytocin-associated neurophysin (p*I* 4.3), could be detected but a species of p$I \sim 4.7$ was found as the major band at all stages except at day 14 where it disappeared. On the other hand, this species was the only one present at days 2 and 3. Assuming that this species might represent the ocytocin-neurophysin common precursor, it was treated with trypsin. This resulted in a dramatic increase of the ocytocin immunoreactivity, i.e. for 2 μg of neurophysin p$I \sim 4.7$ ocytocin immunoreactivity raised from 8.2 ng to 163 ng after trypsin incubation (a 20-fold increase). Moreover, HPLC analysis demonstrated the concomitant appearance of COOH-terminally extended forms of ocytocin, i.e. ocytocin-Gly-Lys-Arg and ocytocin-Gly-Lys, during proteolysis. The evaluated yield of ocytocin-like material (expressed as quantities of ocytocin-Gly-Lys-Arg produced) was above 100%. This value probably reflects an underestimate of pro-ocytocin-neurophysin material since this was determined by using a neurophysin I RIA. Finally, the action of pro-ocytocin convertase from bovine

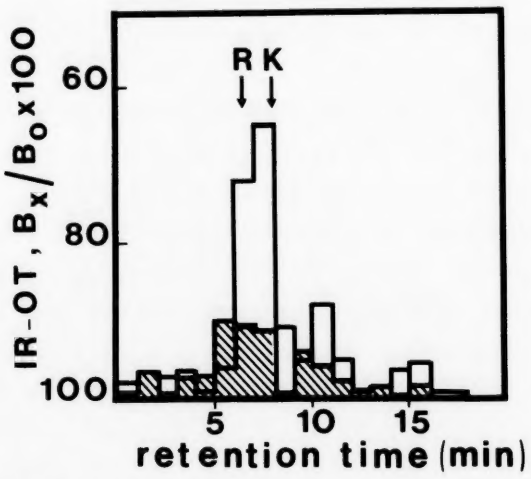

Fig. 2. HPLC analysis of immunoreactive ocytocin (IR-OT) in incubation mixture of the proteins $M_r \sim 10{,}000$ from corpus luteum of 2 days after estrus, with pro-ocytocin convertase (open bars). LKB apparatus was equipped with a Nucleosil 5 CN column (200×4 mm) (Macherey Nagel, Düren, F.R.G.). Elution was carried out with 10% acetonitrile in 0.05% trifluoroacetic acid. Flow rate: 0.6 ml/min. Proteins $M_r \sim 10{,}000$ without enzyme (hatched bars) and pro-ocytocin convertase alone were also analyzed. Pro-ocytocin convertase is not shown because it was free of immunoreactive ocytocin. Arrows indicate the retention times of the following species: R, ocytocin-Gly-Lys-Arg; K, ocytocin-Gly-Lys. B_x and B_0 represent the amount of antibody bound [^{125}I]ocytocin in the presence or in the absence of competitor respectively.

neurohypophysis on this p$I \sim 4.7$ neurophysin under the incubation conditions used to test this endoprotease (Clamagirand et al., 1987a) resulted in both an increase of ocytocin immunoreactivity and the appearance of ocytocin-Gly-Lys-Arg and ocytocin-Gly-Lys (see Fig. 2). Together these data identify the p$I \sim 4.7$ species as the common precursor to ocytocin and neurophysin. Noticeably, its behavior on HPLC was undistinguishable from that of the hemisynthetic precursor prepared by Brakch et al. (1989).

From days 4–5, other neurophysin species appeared (Fig. 1) which were also found at days 7–8 and in extracts of corpora lutea collected from abattoir cows slaughtered at luteal stage II. These species could derive from neurophysin I since ocytocin begins to be detectable at this stage (Fehr et al., 1987) indicating that processing of the prohormone is set up and generates neurophysin I.

In order to study initiation of the post-translational processing of pro-ocytocin-neurophysin, granulosa cells in culture were now chosen as starting material. Primary cultures of luteinizing granulosa cells provide a good model to study expression and regulation of genes coding both for pro-ocytocin-neurophysin, pro-ocytocin convertase and associated enzymes (Clamagirand et al., 1986; Norenberg and Richter, 1988). Whereas production of immunoreactive ocytocin and neurophysin in culture media appeared to be low when granulosa cells were cultured in serum-free medium containing insulin, this production was only 3- to 4-fold stimulated by both testosterone and dihydrotestosterone while follitropin (FSH) was very efficient (10- to 100-fold increase) as noted by Schams (1987). Secretion of progesterone, a marker of luteinization, was also dramatically increased. Fig. 3 shows that, whereas in controls both ocytocin and neurophysin secretions were below 0.1 nmol/l per 10^5 cells per 24 h, during the culture in stimulated media they reached 0.5 nmol as soon as day 2, and attained a level of 1–1.5 nmol/l per 10^5 cells per 24 h at days 3–5.

The immunoreactive neurophysins consisted of ocytocin-neurophysin precursor at p$I \sim 4.7$ and of two less acidic proteins than neurophysin I in smaller quantities (Fig. 1), a result comparable with those of corpus luteum extracts at days 4–5 and 7–8. The immunoreactive ocytocin was con-

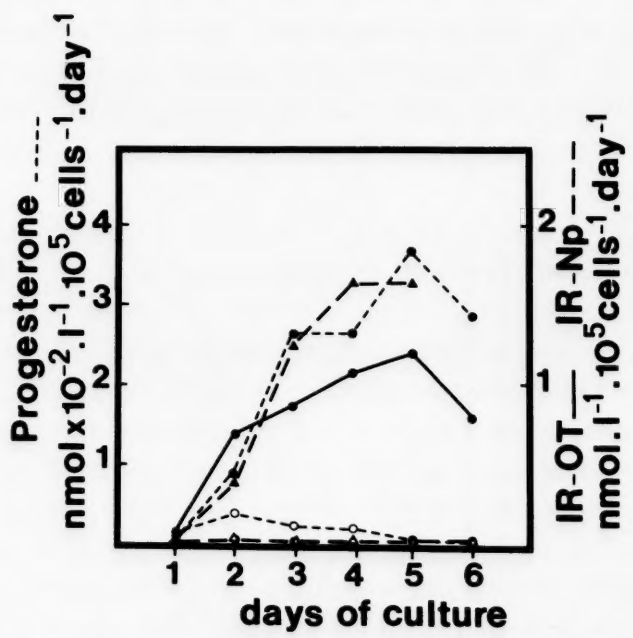

Fig. 3. Secretion of immunoreactive ocytocin (IR-OT), immunoreactive neurophysin (IR-Np) and progesterone by bovine granulosa cells cultured in the presence (●, ▲, ●) and absence (○, △, ○) of FSH and testosterone. Both cultures and RIA for each value were made in triplicate.

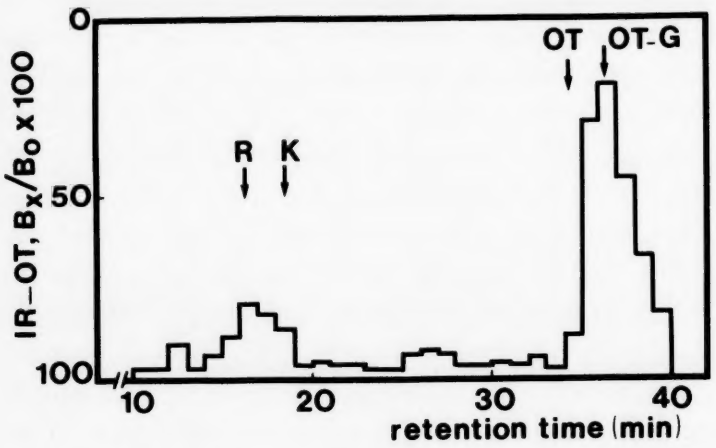

Fig. 4. HPLC analysis of immunoreactive ocytocin (IR-OT) released by FSH, testosterone-stimulated granulosa cells into culture medium at day 3. Beckman apparatus was equipped with a Partisil ODS 3 column (250×4.6 mm) (S.F.C.C., Neuilly-Plaisance, France). Ocytocin peptides previously extracted using Sep-Pak C-18 cartridge were eluted with 18% acetonitrile in 0.01% trifluoroacetic acid at 0.6 ml/min. Arrows indicate the retention times of the following peptides: R, ocytocin-Gly-Lys-Arg; K, ocytocin-Gly-Lys; OT, ocytocin-NH_2; OT-G, ocytocin-Gly. B_x et B_0 represent the amount of antibody bound [^{125}I]ocytocin in the presence or in the absence of competitor respectively.

stituted by ocytocin-Gly-Lys-Arg, ocytocin-Gly-Lys and ocytocin-Gly, whereas ocytocin, the fully mature and amidated peptide, was not detected (Fig. 4). Since the antibodies used in this assay better recognized mature ocytocin than the COOH-terminally extended forms of the peptide (see Materials and Methods), this clearly demonstrates the absence of this species in the culture medium, an observation in keeping with reports by Plevrakis et al. (1990) on human granulosa cells.

Discussion

Previous reports indicated a lag phase between pro-ocytocin-neurophysin mRNA transcription and the appearance of mature ocytocin in newly formed corpus luteum (Ivell et al., 1985; Fehr et al., 1987). Our results demonstrate the presence of pro-ocytocin-neurophysin in the early corpus luteum. Therefore we can propose that pro-ocytocin-neurophysin mRNA translation takes place soon after transcriptional expression of the gene, but that processing is not working or is very low. This may be compared with a report by Wathes et al. (1986) of the cytoimmunochemical detection of neurophysin in granulosa-derived cells from the new corpus luteum.

At days 4–6 after estrus, pro-ocytocin-neurophysin mRNA level is still high and ocytocin production is already significant (Fehr et al., 1987). Our observations indicate the presence of mature neurophysin species besides the intact precursor, suggesting that processing is taking place. After this period, the percentage of ocytocin-neurophysin mRNA is lowered but ocytocin production appears to increase. This can be explained by the fact that, in the developing corpus luteum, immunoreactive ocytocin is indeed constituted by intermediary forms generated by pro-ocytocin-neurophysin processing, whereas amidated ocytocin would be only produced by the mature corpus luteum. Since these COOH-terminally extended forms of ocytocin which are detected in extracts of corpus luteum (Clamagirand et al., 1987a) and in bovine granulosa cell culture were not recognized by antibodies selective for mature ocytocin, i.e. possessing a terminal $CONH_2$, this led to an underestimate of the ocytocin material. The same biosynthetic intermediates were also characterized in human granulosa cells culture (Plevrakis et al., 1990). Together these data indicate that processing is incomplete. This could be due to cofactor requirements for the terminal amidating enzyme which transforms ocytocin-Gly in ocytocin-NH_2. It is clear that ascorbate is mandatory for the activity of the amidating enzyme (Eipper et al., 1983) and that a significant activation of ocytocin-NH_2 production and secretion can be obtained by addition of this agent to cell cultures (Luck and Jungclas, 1988; Plevrakis et al., 1990). According to Sheldrick and Flint (1989) the content in ascorbate of the early corpus luteum 'in vivo' is sufficient for the amidating enzyme to function but the amidation activity increases concomitantly with the development of the corpus luteum. Therefore, it is likely that other regulation(s) may exist at the level of the amidation step.

We propose that post-translational processing of pro-ocytocin-neurophysin in the bovine corpus luteum is set up progressively with a correlative release of COOH-extended ocytocin forms. Although these peptides have been at first considered as intermediary forms of processing they

might turn out, indeed, to play a role in the development of the corpus luteum.

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Cytochalasin B stimulates insulin-like growth factor-binding protein-1 production by Hep G2 cells

Moira S. Lewitt and Robert C. Baxter

Department of Medicine, University of Sydney, and the Department of Endocrinology, Royal Prince Alfred Hospital, Camperdown, New South Wales, Australia

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Key words: Insulin-like growth factor; Insulin-like growth factor-binding protein; Cytochalasin B; Glucose uptake; Glucose homeostasis; (Hep G2)

Summary

Hep G2 cells were used to study the early sequence of events regulating production of insulin-like growth factor-binding protein-1 (IGFBP-1). Cytochalasin B (100 μM) specifically inhibited 2-deoxyglucose uptake by Hep G2 cells and stimulated IGFBP-1 production 2-fold. Insulin (300 nM) did not stimulate hexose uptake but inhibited IGFBP-1 production more than 50%. A change in IGFBP-1 secretion was observed as early as 2 h after a 15-min or 2-h pulse exposure to either effector. In contrast to IGFBP-1, albumin production was diminished in the presence of cytochalasin B and increased by insulin. From these results we conclude that IGFBP-1 synthesis is (i) stimulated by transient inhibition of cellular glucose uptake and further stimulated by long-term glucose deprivation, and (ii) inhibited by transient exposure to insulin with further inhibition on long-term exposure. These effects are consistent with the dynamic regulation of IGFBP-1 by nutritional status.

Introduction

The insulin-like growth factors (IGFs) are polypeptides which are closely related to insulin in structure and activities (Baxter, 1986). They have insulin-like effects as well as growth-promoting actions (Froesch et al., 1985; Baxter, 1986). The IGFs in the circulation represent a considerable hypoglycemic potential (Guler et al., 1987, 1989) which is thought to be blocked by their association with binding proteins. The nomenclature of the IGF-binding proteins has recently been standardized, with the abbreviations IGFBP-1, IGFBP-2 and IGFBP-3 for the three classes for which cDNAs have been cloned and sequenced (Ballard et al., 1990). IGFBP-3 circulates as a growth hormone (GH)-dependent complex of approximately 140 kDa and appears to be the predominant physiological carrier of the IGFs (Baxter and Martin, 1986, 1989). IGFBP-2 has been isolated from rat and bovine cell cultures and shows a selective affinity for IGF-II (Mottola et al., 1986; Szabo et al., 1988). Its function in man is unknown, but its regulation in rodents has been partially characterized with the observation that its expression is increased in adult rat liver during

Address for correspondence: Dr. Moira S. Lewitt, Department of Medicine, University of Sydney, Sydney, NSW 2006, Australia.

starvation or hypophysectomy (Margot et al., 1989; Orlowski et al., 1990).

IGFBP-1 is a GH-independent protein isolated from amniotic fluid and human deciduum. It has previously been known as BP-28 (Baxter et al., 1987), amniotic fluid binding protein (Póvoa et al., 1984), placental protein 12 (Bohn and Kraus, 1980; Julkunen et al., 1988), IBP-1 (Brinkman et al., 1988), IGF BP-25 (Lee et al., 1988) and pregnancy-associated endometrial α_1-globulin (Bell and Keyte, 1988). The full primary structure of this protein has been predicted on the basis of cDNA sequencing in several laboratories (Brewer et al., 1988; Brinkman et al., 1988; Julkunen et al., 1988; Lee et al., 1988). Specific mRNA expression has been reported for a range of tissues by two of these groups. Significant levels of IGFBP-1 mRNA expression are found in liver, secretory endometrium and pregnancy deciduum (Brinkman et al., 1988; Julkunen et al., 1988). There is specific mRNA expression by some human breast cancer cells and by Hep G2 human hepatoma cells, and the protein product is found in the conditioned medium of both of these groups of immortalized cell lines (Póvoa et al., 1985; Julkunen et al., 1988; Lee et al., 1988; Yee et al., 1989).

Patterns of IGFBP-1 regulation in vivo indicate dynamic control by metabolic status. It appears to be modulated by substrate availability as it is suppressed by food intake, specifically by glucose ingestion (Busby et al., 1988; Cotterill et al., 1988; Yeoh and Baxter, 1988), and increased in response to starvation and hypoglycemia (Busby et al., 1988; Cotterill et al., 1988; Hall et al., 1988; Jeoh and Baxter, 1988). Exogenous insulin inhibits circulating IGFBP-1 levels when euglycemia is maintained during glucose clamping (Brismar et al., 1988; Holly et al., 1988; Suikkari et al., 1988). Prolonged exercise increases IGFBP-1 concentrations (Suikkari et al., 1989a) and levels are suppressed in Cushing's disease (Degerblad et al., 1989). These responses resemble those of counterregulatory hormones (Cryer, 1981). Studies in vitro have demonstrated a pattern of IGFBP-1 production which closely parallels the regulation in vivo. We have previously reported a tissue culture model for IGFBP-1 synthesis using human fetal liver, and showed that a cyclic nucleotide-dependent pathway, activated by blocking hexose transport and inhibitable by insulin, appears to be involved in initiating IGFBP-1 synthesis (Lewitt and Baxter, 1989, 1990). Production of IGFBP-1 by granulosa-luteal cells is regulated both via protein kinase-C- and adenylate cyclase-dependent pathways (Jalkanen et al., 1989).

If IGFBP-1 has a counterregulatory role, as previously suggested (Yeoh and Baxter, 1988), it must respond rapidly to metabolic and hormonal changes. A continuous cell line is a convenient model for examining the early sequence of events in IGFBP-1 production. Hep G2 is a human hepatoma-derived cell line that maintains gross liver cell morphology and retains the specialized functions of liver parenchymal cells such as albumin synthesis (Aden et al., 1979; Knowles et al., 1980). It has previously been observed that IGFBP-1 production by Hep G2 cells is suppressed by insulin (Conover et al., 1989; Cotterill et al., 1989; Conover and Lee, 1990; Singh et al., 1990) but there have been conflicting data regarding the effect of varying glucose concentrations (Cotterill et al., 1989; Conover and Lee, 1990). In this paper we demonstrate the stimulatory effect of cytochalasin B, a specific inhibitor of hexose uptake in various tissues (Jung and Rampal, 1977; Axelrod and Pilch, 1983; Ciaraldi et al., 1986; Lewitt and Baxter, 1990), on production of IGFBP-1 by Hep G2 cells. Furthermore we examine the effect of transient exposure to cytochalasin B and insulin on the subsequent short-term production of IGFBP-1 in vitro.

Materials and methods

Cell culture

Hep G2 human hepatoma cells were obtained from ATCC (Rockville, MD, U.S.A.). Cells were maintained in Eagle's minimal essential medium (MEM; Sigma, St. Louis, MO, U.S.A.) supplemented with 10% fetal calf serum (Cytosystems, Castle Hill, Australia) and subcultured at a 1 : 5 split ratio every 5–7 days. For experiments cells were plated in 12-place multiwells (Costar, Cambridge, MA, U.S.A.) and maintained in a humidified incubator. At confluence the medium was changed to MEM plus 0.2% bovine serum albumin (BSA) (RIA grade, Sigma) for 24 h before the experimental additions. The experimental incubations were in 1–2 ml of MEM plus 0.2% BSA. In

experiments designed to evaluate the effect of transient exposure to cytochalasin B or insulin on subsequent IGFBP-1 production, cells were preincubated with cytochalasin B or insulin for the times indicated. Wells were then washed twice with phosphate-buffered saline (PBS) and the medium replaced without cytochalasin B or insulin, representing transient exposure; or with cytochalasin B or insulin, representing continuous exposure. Control wells were also washed after 2 h and the medium replaced. During time-course experiments 120 μl were sampled at 2 h and 4 h from an original volume of 2 ml. Conditioned medium was stored at −20°C for measurement of IGFBP-1 and albumin levels.

Reagents

Cytochalasin B, cytochalasin E, porcine insulin, cycloheximide and actinomycin D were obtained from Sigma. Cytochalasins were dissolved in dimethyl sulfoxide (100 mg/ml) before use. Reagents were added to the medium in volumes less than 5% of the total. Purification of IGFBP-1 from amniotic fluid has been described previously (Baxter et al., 1987). Human crystalline albumin was purchased from Calbiochem (San Diego, CA, U.S.A.). Iodinations of IGFBP-1 and human albumin were performed with chloramine-T (Baxter et al., 1987).

Assays

IGFBP-1 levels were measured by a specific radioimmunoassay (RIA) as described previously (Baxter et al., 1987) with a slight modification (Lewitt and Baxter, 1990). In brief, a specific rabbit antiserum, designated A2, was used at a final dilution of 1:50,000. The effective assay range was 10–1000 ng/ml. Samples from one experiment were assayed together using 10–50 μl conditioned medium. The within-assay imprecision for 20 μl samples was 8.7% at 73 ng/ml and 9.7% at 302 ng/ml. Albumin levels were measured by a specific RIA described previously (Lewitt and Baxter, 1989). In brief, a rabbit antiserum against human albumin (Behringwerke, Marburg-Lahn, F.R.G.) was used at a final dilution of 1:12,500. Samples from one experiment were assayed together using 10–50 μl conditioned medium. The within-assay imprecision for 10 μl samples was 4.2% at 1.6 μg/ml and 4.3% at 11.8 μg/ml. The total cell protein content of representative confluent wells was measured by the Lowry modification of the Folin assay (Lowry et al., 1951). The between-plate coefficient of variation of protein content ranged from 5 to 10% over three experiments. The pattern of results presented in this paper was not altered by correcting for protein content and results are therefore expressed as nanograms of IGFBP-1 or micrograms of albumin per well. When comparing IGFBP-1 and albumin production rates, molecular masses were assumed to be 25 kDa and 69 kDa respectively.

2-Deoxyglucose uptake

Cells were plated in 12- or 24-place multiwells and used at confluence after 24-h serumfree. Uptake of 0.5 μCi/ml 2-deoxy-D-[2,6-^{3}H]glucose (42 Ci/mmol; Amersham, Bucks, U.K.) was determined. For dose–response experiments using cytochalasin B, cytochalasin E or insulin, cells were placed in PBS for 2 h and then incubated with 2-deoxy-D-[2,6-^{3}H]glucose in 1 ml PBS for the times indicated. In experiments designed to evaluate the effect on subsequent glucose uptake of a transient exposure to cytochalasin B, cells were preincubated in MEM plus 0.2% BSA with or without cytochalasin B for the times indicated. Wells were then washed twice with PBS and for half the uptake of 2-deoxy-D-[2,6-^{3}H]glucose was measured immediately in 1 ml PBS during a 15-min incubation in the absence of cytochalasin B. For the remaining wells the medium was replaced without cytochalasin B for a 2-h recovery period before 2-deoxyglucose uptake was determined. For all experiments uptake was inhibited by three rapid washes with cold normal saline containing 30 mM glucose. The cells were then dissolved in 500 μl 0.1% sodium dodecyl sulfate and radioactivity determined with a liquid scintillation counter using 5 ml of Picofluor (Canberra Packard, Zurich, Switzerland). Results are expressed as counts per min/well.

Statistics

Each experiment presented in this paper has been repeated on at least three occasions with similar results. Values represent the mean ± SE of 4–8 replicates in a single experiment. Unless otherwise indicated statistical analysis was carried

out by analysis of variance and Duncan's multiple range test (Bruning and Kintz, 1968).

Results

IGFBP-1 was produced by Hep G2 cells at approximately 1 μg/mg protein/24 h and was inhibited 84% after 24 h exposure to 1.5 μg/ml cycloheximide or 70% during a 6-h incubation with 0.1 μg/ml actinomycin D, an inhibitor of DNA transcription (data not shown). Fig. 1 shows IGFBP-1 production by Hep G2 cells during a 24-h period. IGFBP-1 production was increased by cytochalasin B, a specific inhibitor of hexose transport (Jung and Rampal, 1977; Axelrod and Pilch, 1983; Ciaraldi et al., 1986; Lewitt and Baxter, 1990), in a dose-dependent manner (Fig. 1*A*). Because of limited solubility of the cytochalasins, a final concentration of 0.3% dimethyl sulfoxide was required to keep 100 μM cytochalasin B in solution. This concentration of dimethyl sulfoxide did not alter IGFBP-1 production compared to control plates (data not shown). For pooled data from four experiments, 10 μM cytochalasin B stimulated IGFBP-1 production by Hep G2 cells during 24 h to $139 \pm 2\%$ of control plates ($P < 0.001$; paired t-test). In three experiments 100 μM cytochalasin B stimulated IGFBP-1 $224 \pm 21\%$ compared to control plates ($P < 0.05$; paired t-test). We also confirmed that insulin inhibited IGFBP-1 production in a dose-dependent manner. For pooled data from three experiments, 3 nM insulin inhibited IGFBP-1 secretion during 24 h to $60 \pm 11\%$ of control ($P = 0.064$; paired t-test). In four experiments 300 nM insulin inhibited IGFBP-1 to $43 \pm 8\%$ of control plates ($P < 0.01$; paired t-test).

To confirm that cytochalasin B inhibits hexose uptake by Hep G2 cells and to determine the effect of insulin, the uptake of a nonmetabolized sugar, 2-deoxyglucose, was measured in the presence of these agents. Cytochalasin E, a compound related to cytochalasin B, which does not inhibit hexose transport in other systems (Jung and Rampal, 1977; Axelrod and Pilch, 1983; Lewitt and Baxter, 1990), was used as a control. Uptake of 2-deoxyglucose was linear in control wells over a 60-min time course (data not shown). Fig. 1*B* shows the effect of increasing concentrations of cytochalasin B and cytochalasin E on 2-deoxy-D-[^{3}H]glucose uptake over 15 min by Hep G2 cells. Compared to control plates, 15-min uptake was inhibited 95% by 100 μM cytochalasin B ($P < 0.001$) and not influenced by 100 μM cytochalasin E. It was noted, however, that with more prolonged incubations 2-deoxyglucose uptake was sometimes impaired in the presence of high concentrations of cytochalasin E, presumably indicating a nonspecific effect common to all cytochalasins. Uptake of 2-deoxyglucose over 15 min in the

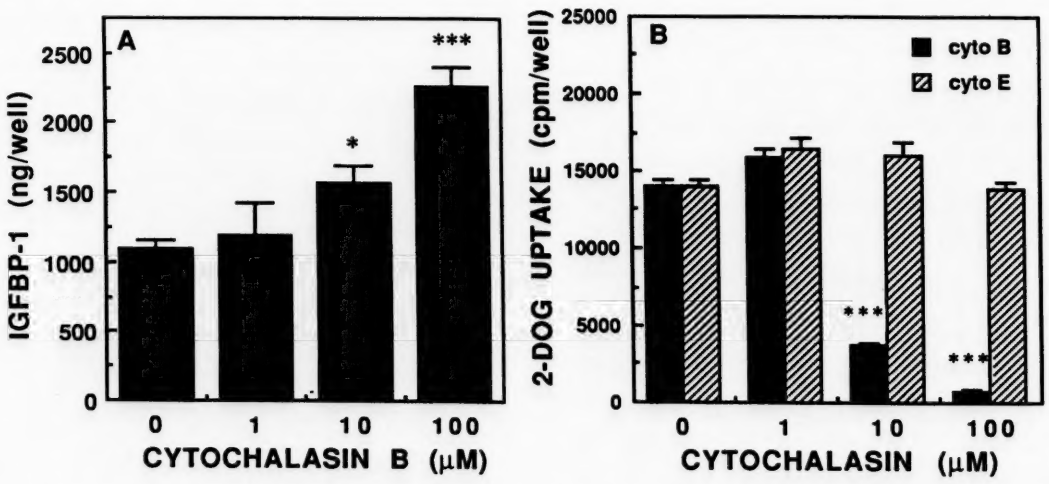

Fig. 1. *A*: Stimulation by cytochalasin B of IGFBP-1 production by Hep G2 cells during a 24-h incubation. Values are expressed as the mean $\pm$ SE for four wells. * $P < 0.05$; *** $P < 0.001$ (compared to control wells). *B*: Effect of cytochalasins on uptake of 2-deoxy-D-[^{3}H]glucose (2-DOG) by Hep G2 cells during a 15-min incubation. Each well was incubated with 2-DOG, 0.5 μCi/ml in phosphate-buffered saline and increasing concentrations of cytochalasin B (cyto B) or cytochalasin E (cyto E) for 15 min. Uptake was terminated by three washes with cold normal saline containing 30 mM glucose. Values are expressed as the mean $\pm$ SE for six wells. *** $P < 0.001$ (compared to control wells).

presence of 300 nM insulin was not different from control plates (data not shown).

If IGFBP-1 has a dynamic role in regulating IGF availability then regulation of its production would need to occur quickly. Fig. 2 demonstrates that the effect of cytochalasin B and insulin can be observed within hours of their presentation to Hep G2 cells. In this experiment cells were preincubated with 100 μM cytochalasin B or 300 nM insulin for 2 h, washed twice, then changed to medium not containing either effector, representing a 'pulse' stimulus. Control wells were also washed after 2 h and medium replaced. Samples were obtained 2, 4 and 6 h after the medium change. Significant stimulation by cytochalasin B was observed within 2 h of a pulse stimulus ($P < 0.05$) and suppression by insulin was significant within 4 h ($P < 0.001$). Production of albumin, a secreted product of this hepatoma-derived cell line (Knowles et al., 1980), was inhibited by cytochalasin B and increased by insulin. Cytochalasin E was used as a control for the nonspecific toxic effect of the cytochalasins. Following a 2-h pulse exposure to 100 μM cytochalasin E, production of

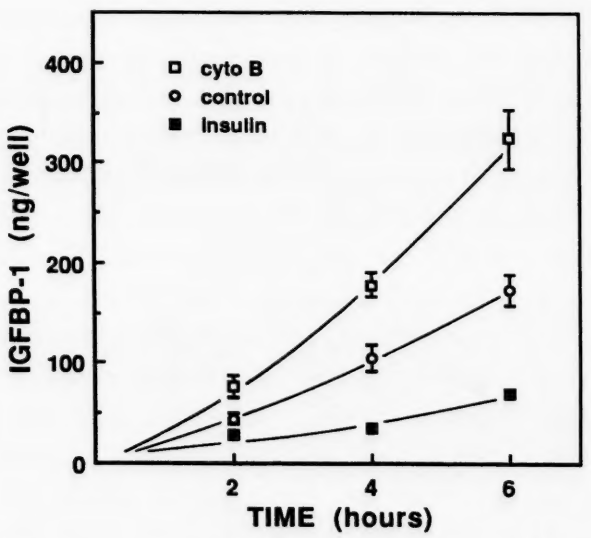

Fig. 2. Time course of IGFBP-1 stimulation by cytochalasin B and inhibition by insulin in Hep G2 cells. Cells were preincubated in the presence and absence of 100 μM cytochalasin B (cyto B) or 300 nM insulin for 2 h. Wells were then washed twice in phosphate-buffered saline and changed to medium not containing cytochalasin B or insulin. Values are expressed as the mean $\pm$ SE for four or six wells. Compared to control wells, IGFBP-1 levels were higher after exposure to cytochalasin B at 2 h ($P < 0.05$), 4 h ($P < 0.01$) and 6 h ($P < 0.001$). IGFBP-1 was lower compared to controls after exposure to insulin ($P < 0.001$ at 4 h and 6 h).

TABLE 1

EFFECT OF 2-h PREINCUBATION WITH CYTOCHALASIN B, CYTOCHALASIN E AND INSULIN ON SUBSEQUENT IGFBP-1 AND ALBUMIN PRODUCTION DURING A 6-h INCUBATION

Cells were preincubated with 100 μM cytochalasin B, 100 μM cytochalasin E or 300 nM insulin for 2 h. They were then washed twice in phosphate-buffered saline and changed to medium not containing either effector, representing a 'pulse' stimulus. IGFBP-1 and albumin levels were measured 6 h after the medium change. Values are expressed as a percentage of control wells and are the mean $\pm$ SE pooled for five experiments using cytochalasin B, two experiments using cytochalasin B and three experiments using insulin.

	IGFBP-1 (%)	Albumin (%)
Cytochalasin B 100 μM	193 $\pm$ 36 [a]	83 $\pm$ 7
Cytochalasin E 100 μM	71 $\pm$ 1 [a]	79 $\pm$ 11
Insulin 300 nM	48 $\pm$ 5 [b]	115 $\pm$ 11

[a] $P < 0.05$ (paired t-test).
[b] $P < 0.01$ (paired t-test).

IGFBP-1 and albumin was inhibited and the molar ratio of IGFBP-1 to albumin production was not significantly different from controls. The results for five experiments using cytochalasin B, two experiments using cytochalasin E and three experiments using insulin were pooled and are shown in Table 1.

Since exposure to a 2-h pulse was effective in modulating IGFBP-1 production by Hep G2 cells we looked at shorter exposure times. In Fig. 3*A* the effect on IGFBP-1 secretion following a 15-min preincubation with 100 μM cytochalasin B and 300 nM insulin is compared to a 2-h preincubation and to the continuous presence of these agents. Cells were preincubated for 15 min or 2 h with cytochalasin B or insulin and then either placed in medium without cytochalasin B or insulin, representing a 15-min or 2-h 'pulse' exposure; or with cytochalasin B or insulin replaced, representing a 'continuous' stimulus. There was a small stimulatory effect of 100 μM cytochalasin B on the production of IGFBP-1 following a 15-min exposure, not reaching statistical significance. The stimulatory effect of cytochalasin B was significant after a 2-h exposure ($P < 0.01$) and was not different from the effect on cells continuously exposed to cytochalasin B. The inhibitory effect of insulin on the production of IGFBP-1 was seen after only a 15-min exposure to 300 nM insulin

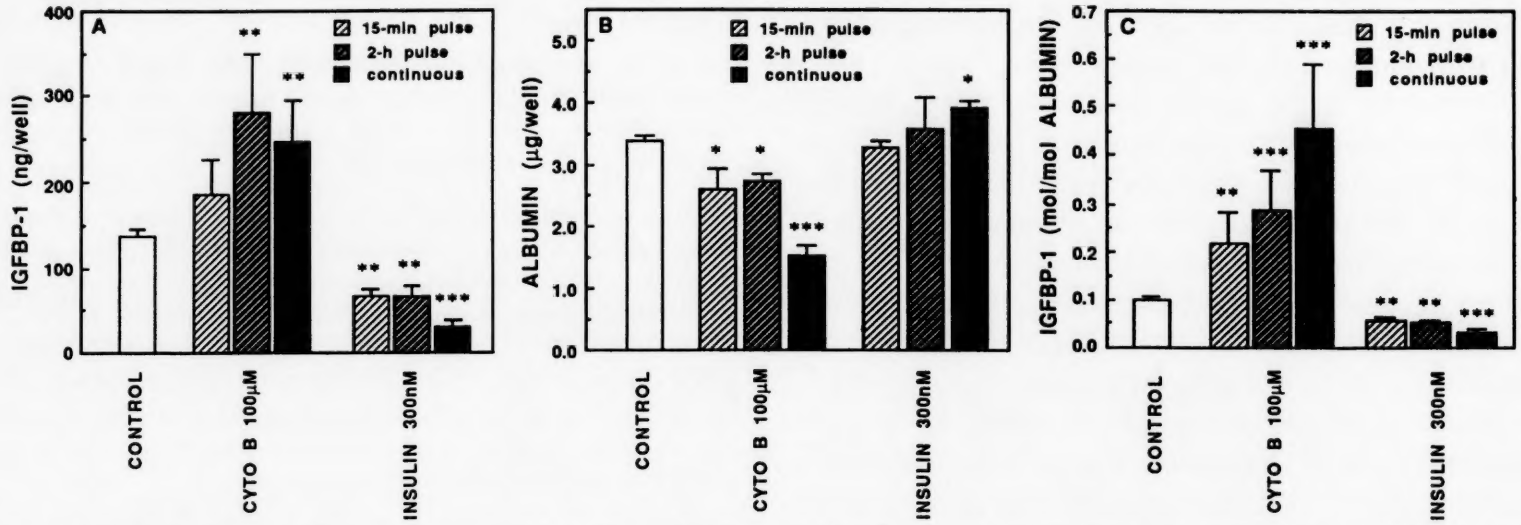

Fig. 3. Effect of varying the time of exposure to cytochalasin B and insulin on the subsequent 6-h production of IGFBP-1 and albumin by Hep G2 cells. Cells were incubated in the presence and absence of 100 μM cytochalasin B (CYTO B) or 300 nM insulin for 15 min or 2 h. Cells were then washed twice with phosphate-buffered saline. The added effectors were replaced in some of the wells, representing a 'continuous' stimulus. The remaining wells were changed to medium not containing cytochalasin B or insulin, representing a 15-min or 2-h 'pulse' stimulus. IGFBP-1 production during the subsequent 6 h is shown in panel *A*. Albumin production in the same experiment is shown in panel *B*. In panel *C* IGFBP-1 production is expressed as mol/mol albumin production. Values are expressed as mean $\pm$ SE for four wells. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

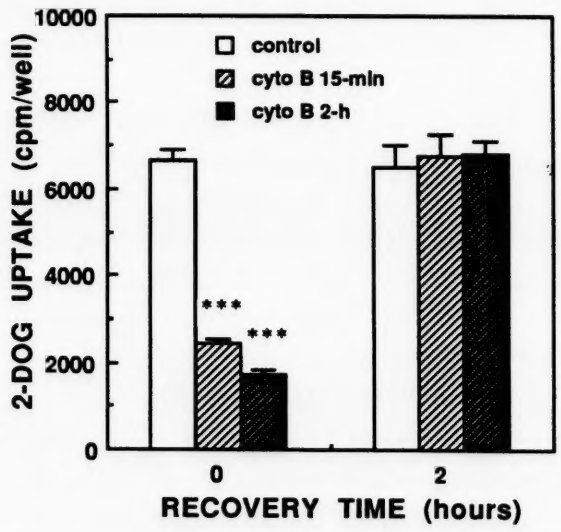

Fig. 4. Recovery of hexose uptake by Hep G2 cells following transient exposure to cytochalasin B. Cells were incubated in the presence and absence of 100 μM cytochalasin B (cyto B) for 15 min or 2 h. Wells were then washed twice with phosphate-buffered saline and, for half the wells, uptake of 2-deoxy-D-[^{3}H]glucose (2-DOG) was determined immediately. The rest of the wells were changed to medium not containing cytochalasin B and 2-DOG uptake determined after a further 2-h incubation period. Each well was incubated with 2-DOG, 0.5 μCi/ml in phosphate-buffered saline and at 15-min uptake was terminated by three washes with cold normal saline containing 30 mM glucose. Values are expressed as the mean $\pm$ SE for eight wells. *** $P < 0.001$ (compared to control wells).

($P < 0.01$) and did not differ from the effect of a 2-h exposure. There was further suppression of IGFBP-1 in the continuous presence of insulin ($P < 0.001$). In Fig. 3*B*, the albumin production in 6 h is shown for the same experiment. In contrast to IGFBP-1, albumin was slightly inhibited by preincubation with 100 μM cytochalasin B ($P < 0.05$) and even more so by the continuous presence of cytochalasin B ($P < 0.001$), presumably representing a nonspecific effect of cytochalasins on cell protein synthesis. The continuous presence of insulin slightly stimulated albumin production by Hep G2 hepatoma cells ($P < 0.05$).

On the assumption that albumin production rates might reflect 'general' protein synthesis by the cells, the IGFBP-1 production rate by each well was expressed relative to albumin production rate (Fig. 3*C*). Whereas under basal conditions the rate of IGFBP-1 synthesis was only 10% that of albumin, this was increased to more than 20% by even a 15-min exposure to cytochalasin B. In the presence of continuous exposure to cytochalasin B, IGFBP-1 was synthesized at almost 50% the rate of albumin. A 15-min exposure to insulin reduced the IGFBP-1 production rate of 5% that of albumin, and continuous exposure decreased this to less than 3%.

Since even a transient 15-min or 2-h exposure

to cytochalasin B was able to increase IGFBP-1 production compared to albumin production, it was important to establish that the inhibition of glucose uptake in these experiments was indeed transient. Therefore we measured 2-deoxyglucose uptake immediately following, and 2 h after, 15-min and 2-h pulse exposures to 100 μM cytochalasin B. Results of a representative experiment are shown in Fig. 4. Although 15-min and 2-h exposures to 100 μM cytochalasin B immediately inhibited 2-deoxyglucose uptake to 37% and 26% of control values, the uptake rates had recovered to normal 2 h after removing cytochalasin B. This confirms that pulse exposure to cytochalasin B results in transient inhibition of hexose transport, and indicates that this transient substrate deprivation is sufficient to stimulate IGFBP-1 production over a 6-h period.

Discussion

These in vitro studies demonstrate that IGFBP-1 production by Hep G2 human hepatoma cells is stimulated in short-term experiments by cytochalasin B, an inhibitor of glucose uptake. Insulin did not modulate glucose uptake but, as previously documented, suppressed IGFBP-1 production. Changes in secreted protein levels can be observed as early as 2 h after application of a stimulus, with only a transient blocking of hexose uptake or transient exposure to insulin needed to effect a response.

Both the nature and time course of the stimulatory response appear specific for this protein. In contrast to the increase in IGFBP-1 levels, albumin was inhibited by cytochalasin B. A likely explanation is that while the stimulatory effect of cytochalasin B on IGFBP-1 synthesis is specific, there is also a nonspecific inhibitory effect common to all cytochalasins affecting general protein synthesis, including both albumin and IGFBP-1, and that this nonspecific inhibition is limiting maximal IGFBP-1 production. In support of this explanation we observed that cytochalasin E inhibits both albumin and IGFBP-1 levels such that the molar ratio of IGFBP-1 to albumin production was not altered by cytochalasin E. It is well known that there are other binding sites for cytochalasin B which do not relate to glucose transport and which are bound also by cytochalasin E and other cytochalasins (Jung and Rampal, 1977; Lin and Snyder, 1977; Axelrod and Pilch, 1983). These binding sites probably account for the effects of the cytochalasins on cellular microfilaments (Rathke et al., 1975). Albumin is a major circulating protein and also secreted in substantial quantities by Hep G2 cells. Its production rate may thus provide a measure of 'general' protein synthesis by the cells. For this reason it was useful to compare IGFBP-1 production rates to those of albumin. This indicated that the IGFBP-1 production rate in the basal state is less than 10% of the rate of albumin production. When hexose entry into the cells is blocked, IGFBP-1 production increases considerably to almost 50% of the rate of albumin production. Also in keeping with this concept and consistent with the documented anabolic role for insulin in many cell types, we observed a stimulatory effect of insulin on albumin production. Others have observed that insulin tends to stimulate growth and division of Hep G2 cells (Plymate et al., 1988). Yet insulin suppressed IGFBP-1 production to 3% of the rate of albumin synthesis.

It is well documented that cytochalasin B is a specific inhibitor of the glucose transporter species expressed by human erythrocytes (Jung and Rampal, 1977). The predominant glucose transporter expressed by Hep G2 cells is identical (Mueckler et al., 1985) and in this paper we confirm that cytochalasin B inhibits 2-deoxyglucose uptake in this system. There are conflicting reports of an independent effect of glucose on IGFBP-1 production by Hep G2 cells (Cotterill et al., 1989; Conover and Lee, 1990). In human fetal liver glucose itself inversely regulated IGFBP-1 secretion, but this effect was most consistent in the presence of theophylline (Lewitt and Baxter, 1989) and the effect of cytochalasin B was substantially greater (Lewitt and Baxter, 1990). We also found that Hep G2 cells are not insulin-responsive with respect to glucose uptake, and this has been the observation of another group (Gould et al., 1989). On the other hand one group observed a small stimulatory effect of insulin on hexose uptake (Podskalny et al., 1985). Conover and Lee (1990) have shown, however, by using various monoclonal antibodies to the IGF-I and

insulin receptors, that insulin clearly interacts with its own receptors in Hep G2 cells to suppress IGFBP-1 production. Whether insulin and cytochalasin B act via a common cyclic nucleotide-dependent pathway in Hep G2 cells, as we have suggested for human fetal liver (Lewitt and Baxter, 1990), is yet to be established.

The acute nature of the changes in IGFBP-1 secretion provoked by inhibiting glucose uptake with cytochalasin B and by insulin are in keeping with a dynamic physiological role for this protein. We have proposed that IGFBP-1 has a role in glucose counterregulation (Yeoh and Baxter, 1988). This hypothesis is based on the assumptions that there are free IGFs in the circulation and that IGFBP-1 has an inhibitory effect on IGFs in vivo. Estimates of 'free' IGFs in the circulation approach 3 nM (Guler et al., 1987, 1989) and close regulation of this considerable hypoglycemic potential would seem essential. If indeed IGFBP-1 is able to inhibit the bioactivity of 'free' IGFs, production of IGFBP-1 in response to hypoglycemia or to reduced intracellular substrate availability would allow any 'free' insulin-like activity to be blocked. The concentration range over which IGFBP-1 fluctuates in vivo (from less than 0.4 to 4 nM) would be appropriate to permit this regulation (Yeoh and Baxter, 1988).

With respect to the second assumption, there is considerable in vitro evidence to support an inhibitory effect for IGFBP-1 on the insulin-like and other activities of the IGFs (Drop et al., 1979; Ritvos et al., 1988; Burch et al., 1990). Data in vivo is limited, though it was recently reported that circulating IGFBP-1 is associated with inhibition of serum IGF-I bioactivity (Taylor et al., 1990). It is also interesting to note that an inhibitor of IGF activity appears during insulin-induced hypoglycemia, though apparently only in those patients with a growth hormone response (Hill et al., 1981), and that IGF bioactivity is depressed after glucagon administration (Binoux et al., 1977). We have previously demonstrated that glucagon stimulates IGFBP-1 production by human fetal liver explants (Lewitt and Baxter, 1989). We have also observed a stimulatory effect in Hep G2 cells though of smaller magnitude (data not shown). This counterregulatory hormone may therefore also play an important role in vivo in stimulating IGFBP-1. In contrast epinephrine, another counterregulatory hormone, was not found to be an acute modulator of IGFBP-1 production (Cotterill et al., 1989).

While in vivo glucose clamp studies do not demonstrate an independent effect of varying glucose levels on serum IGFBP-1 levels (Suikkari et al., 1989b), interpretation of these results should take into account that substrate availability is never limited in such experiments as glucose concentrations are maintained by constant glucose infusion in the hyperinsulinemic state. On the other hand it has been shown that if transient hypoglycemia is allowed to occur following bolus administration of insulin, circulating IGFBP-1 levels are stimulated (Yeoh and Baxter, 1988). Our demonstration in vitro that a transient blocking of hexose uptake is sufficient to stimulate hepatic IGFBP-1 production over the following hours is consistent with this phenomenon. We have also shown that a transient exposure to insulin is sufficient to reduce the IGFBP-1 production rate. These findings are consistent with a short-term regulatory function for IGFBP-1 in glucose homeostasis.

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Evidence for regulation of juvenile hormone biosynthesis operating before mevalonate in locust corpora allata

Franck Couillaud

Laboratory of Neuroendocrinology, URA CNRS 1138, University of Bordeaux I, 33405 Talence, France

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Key words: Insect; *Locusta migratoria*; Corpora allata; Mevalonate; Mevalonolactone; Juvenile hormone; Mevinolin; Regulation

Summary

Exogenous mevalonate poorly stimulated juvenile hormone III (JH-3) biosynthesis by corpora allata from *Locusta migratoria*. However, mevalonolactone strongly stimulated glands from different physiological states and fully restored JH synthesis in mevinolin-treated glands. Asymmetry in spontaneous rate of JH release was abolished by exogenous mevalonolactone. After transection of nervus corporis allati 1 (NCA-1), the rate of mevalonolactone-stimulated JH synthesis was maintained at the preoperative levels although the spontaneous rate of JH biosynthesis decreased rapidly. These results suggest that the spontaneous asymmetry of JH biosynthesis and the low rate of JH biosynthesis by denervated corpora allata both result from non-stimulation or inhibition acting on the JH pathway before the utilisation of mevalonate.

Introduction

The mevalonate pathway in insects produces juvenile hormones (JH) which play a crucial role in insect physiology by controlling, for instance, metamorphosis and reproduction (see Feyereisen, 1985; Schooley and Baker, 1985). JH are biosynthesized in small endocrine glands, the corpora allata, under the control of the insect brain. The JH biosynthetic pathway is postulated to be controlled essentially by two mechanisms (Tobe and Pratt, 1976; Feyereisen, 1985): first, the activity of one or few rate-limiting enzymes in the biosynthetic pathway may be regulated; second, the concentration of enzymes involved in the pathway may change under the influence of regulatory factors.

This paper is part of an extensive investigation to localize the rate-limiting enzymes of the JH biosynthesis pathway in the corpora allata of the African locust and examines enzymatic steps subsequent to mevalonate formation. We have taken advantage of two characteristics of JH biosynthesis by locust corpora allata:

First is the large asymmetry in the spontaneous rates of JH biosynthesis by paired locust corpora allata (Couillaud and Girardie, 1987). It has been proposed that this asymmetry of JH production results from an autonomic regulation of each gland involving enzymatic targets (Couillaud and Girardie, 1987). The in vitro stimulation of JH production by exogenous precursors such as farnesol and farnesoic acid suppressed asymmetry.

Address for correspondence: Dr. Franck Couillaud, Laboratoire de Neuroendocrinologie, Université de Bordeaux I, Avenue des Facultés, 33405 Talence Cedex, France.

This suggested that rate-limiting enzymes are located prior to farnesol.

Second is the fact that locust corpora allata isolated from the brain-corpora cardiaca complex exhibit a very low rate of JH biosynthesis (Couillaud and Girardie, 1985). Such induced-low rate of JR production can be stimulated within 3 h when corpora allata are co-incubated with brain extract (Couillaud and Girardie, 1990). This suggests that enzymes involved in JH synthesis are still present in disconnected glands and that the regulation of JH biosynthesis by the factor present in the brain extract is probably acting on the activity of one or few rate-limiting enzymes. Restoration of JH production by exogenous farnesoic acid or farnesol supported a regulation prior to farnesol (Couillaud et al., 1988).

JH-III, the single JH produced by locust corpora allata (Pratt and Weaver, 1978; Mauchamp et al., 1985), is a sesquiterpenoid derived from mevalonate (Schooley and Baker, 1985). Exogenous mevalonate has been shown to serve as JH-III precursor in the corpora allata of several species (Feyereisen, 1985; Schooley and Baker, 1985; Feyereisen and Farnsworth, 1987). The present paper reports the stimulation of JH biosynthesis in unoperated, disconnected and mevinolin-treated corpora allata by mevalonate and mevalonolactone. Our physiological experiments demonstrate that rate-limiting enzymes in this locust are located prior to mevalonate.

Materials and methods

Insects

Locusta migratoria migratorioides were reared at 30°C under crowded conditions. They were fed every morning on fresh wheat and bran. All experimental females were isolated on the first day of adult life. Nervus corporis allati 1 (NCA-1) transection was performed on day 8, using Pascheff Wolff scissors on CO_2-anaesthetized females.

Chemicals

Racemic mevalonolactone (mevalonic acid lactone) from Sigma (Taufkirchen, F.R.G.) was converted to mevalonate by titration with 1 N NaOH. Mevinolin (a potent inhibitor of 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase) was a gift from Dr. A.W. Alberts (Merck Institute for Therapeutic Research, Rahway, NJ, U.S.A.). JH-III was purchased from Calbiochem (San Diego, CA, U.S.A.).

Corpora allata incubations

Glands were incubated at 28°C in TC 199 (Flow Laboratories, Irvine, U.K.) supplemented with Ficoll (20 mg/ml, Sigma, Taufkirchen, F.R.G.) and [^{14}C-*methyl*]methionine (final specific activity 1.5 GBq/mmol, Amersham, Buckinghamshire, U.K.). Racemic mevalonate and mevalonolactone in aqueous solutions were added directly in labelled culture medium. Mevinolin, dissolved in dimethyl sulphoxide (DMSO) was added in incubation medium (final concentration 10 μM, 1% DMSO). Standard incubation time was 3 h. For consecutive incubations, corpora allata were first incubated 30 min with labelled methionine for equilibration and then culture medium, supplemented or not, was changed every hour. Linearity in the spontaneous rate of JH biosynthesis has been published previously (Girardie et al., 1981).

JH biosynthesis

JH biosynthesis was determined using a radiochemical assay according to Pratt and Tobe (1974). The validity of this assay in *Locusta migratoria* has been described extensively (Girardie et al., 1981; Mauchamp et al., 1985; Couillaud et al., 1987). JH-III was separated by silica thin-layer chromatography (TLC) (Merck, Ref. 5735, Darmstadt, F.R.G.) in a xylene/ethyl acetate (4:1) solvent system. Radioactivity of fractions sliced from the TLC plastic plates was determined by liquid scintillation counting with a Beckmann LS 2800 spectrometer (Palo Alto, CA, U.S.A.).

Osmotic pressure measurement

Osmotic pressure was determined at the freezing point using a Knauer osmometer (Berlin, F.R.G.). Changes in osmotic pressure were induced by NaCl addition in incubation medium.

Results

Effect of mevalonate and mevalonolactone on JH biosynthesis

When present in the incubation medium at 120 mM (optimal concentration, result not shown),

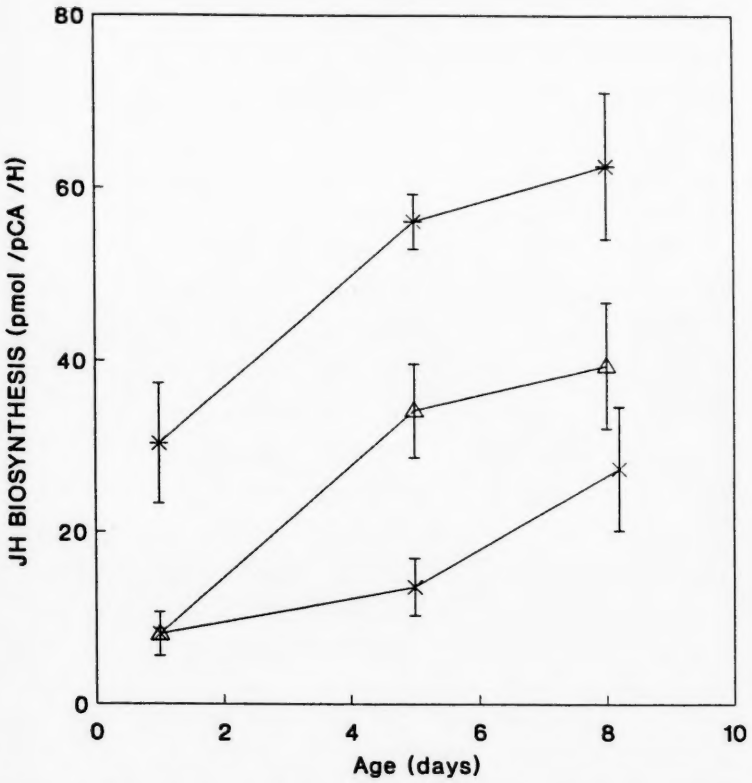

Fig. 1. Spontaneous (×), 120 mM (3*RS*)-mevalonate-stimulated (△) and 120 mM (3*RS*)-mevalonolactone-stimulated (*) rate of JH biosynthesis by corpora allata from 1-, 5- and 8-day-old adult females. Each point (±SEM) represents the mean of 6–10 incubation with single pairs of corpora allata from adult females.

racemic mevalonate stimulated the rate of JH-III biosynthesis by corpora allata from 5-day-old adult females (Fig. 1), but did not stimulate corpora allata from day-1 and day-8 females. However, mevalonic acid lactone added in the incubation medium at the same dose strongly stimulated JH synthesis in corpora allata from the three ages. Mevalonic acid lactone stimulated the rate of JH synthesis 3.7, 4.3 and 2.3 times on days 1, 5 and 8 respectively. On day 5, the mevalonic acid lactone-stimulated rate of JH biosynthesis was 1.6 times higher than mevalonate-stimulated rate.

3-Hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase from *Diploptera* has been shown to produce only (3*R*)-mevalonate (Feyereisen and Farnsworth, 1987) and mevalonate kinase from *Sarcophaga* was found to phosphorylate only one isomer (presumably 3*R*) of a (3*RS*)-mevalonate mixture (Goodfellow et al., 1972). Thus, in the locust, effective concentrations of mevalonate are probably half of the concentrations of racemic mevalonate reported in the present paper.

Changes in pH and osmotic pressure and their effect on JH biosynthesis

During the 3 h incubation, mevalonic acid lactone slowly acidified the medium as revealed by colour changes of phenol red and pH monitoring (Fig. 2). Mevalonate did not modify the pH of the incubation medium. In order to discriminate between the effects of mevalonic acid lactone and of pH, the rate of JH biosynthesis was determined at different pH. Fig. 3 clearly illustrates that a decreasing pH resulted in a significant decrease in JH biosynthesis. Thus, the stimulation of JH biosynthesis by exogenous mevalonic acid lactone cannot be assigned to the acidification of culture medium. In spite of the pH decrease in incubation medium, the mevalonic acid lactone exhibited a strong stimulating effect on JH biosynthesis in vitro.

We also checked changes in osmotic pressure resulting from such high a concentration of mevalonate or mevalonic acid lactone. The typical osmotic pressure for TC 199, as prepared for JH radiochemical assay (see Materials and Methods) is 270 mOsm. Osmotic pressure in mevalonic acid lactone-supplemented medium was 275, 310 and

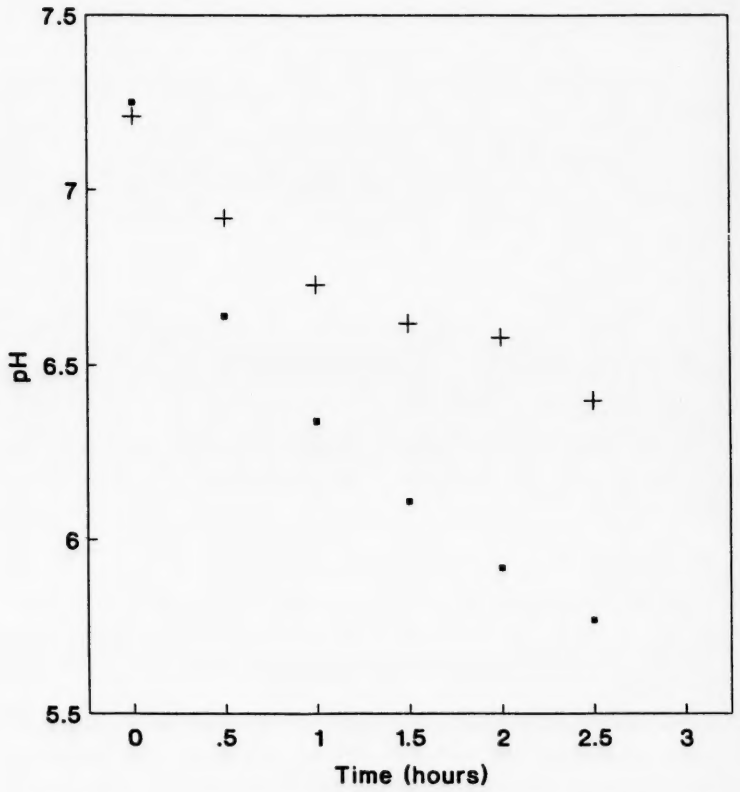

Fig. 2. pH monitoring of incubation medium containing 120 mM (+) or 160 mM (■) mevalonolactone at 30°C during the 3 h incubation time.

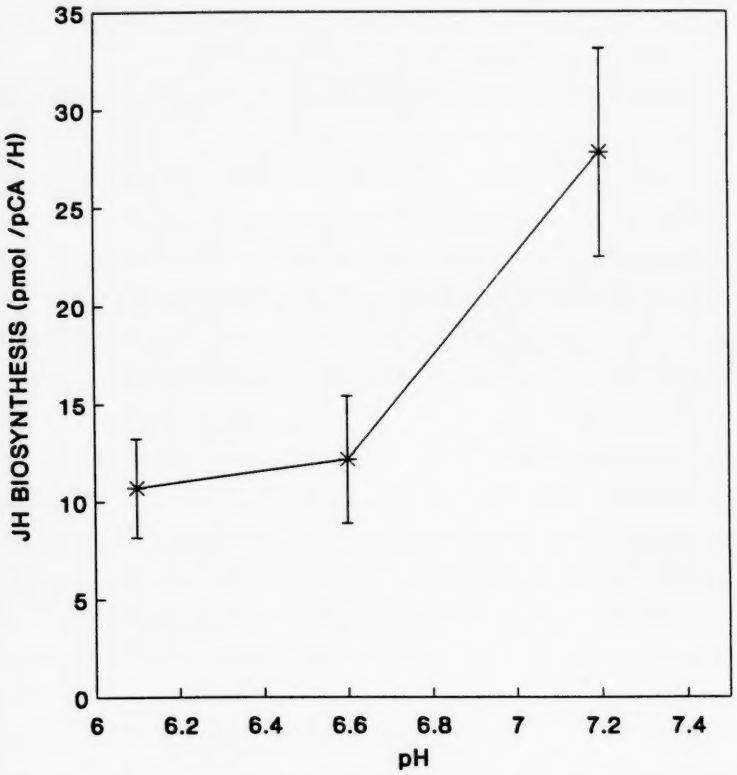

Fig. 3. Influence of the pH of the incubation medium on the rate of JH biosynthesis. Each point (±SEM) represents the mean of 13 incubations (3 h) with single pairs of corpora allata from 8-day-old adult females.

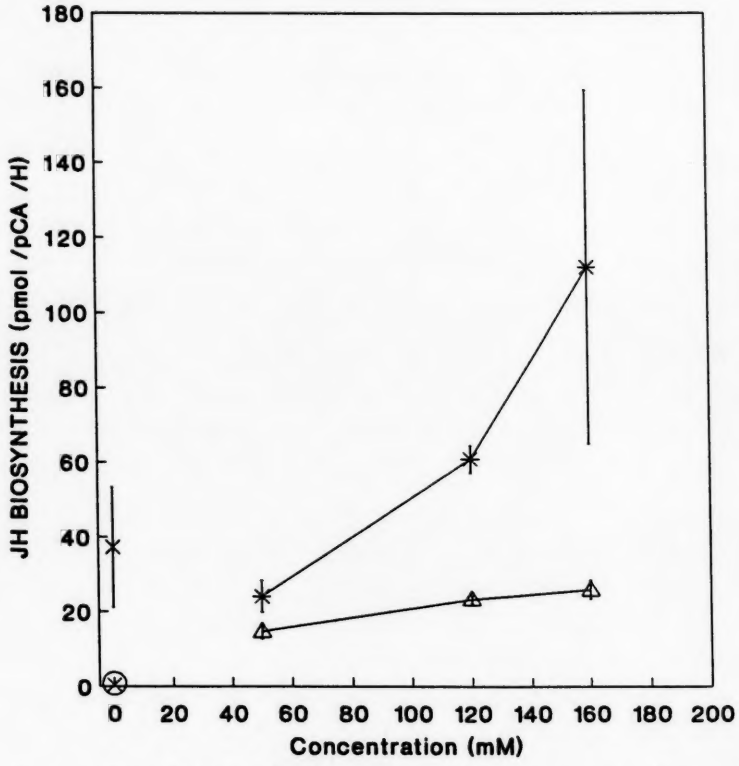

Fig. 5. Stimulation of JH synthesis by mevalonate (△) and mevalonolactone (*) on corpora allata incubated with mevinolin (⊗). Spontaneous activity of untreated gland is shown for comparison (×). Each point (±SEM) represents the mean of 6–10 incubations with single pairs of corpora allata from 8-day-old adult females.

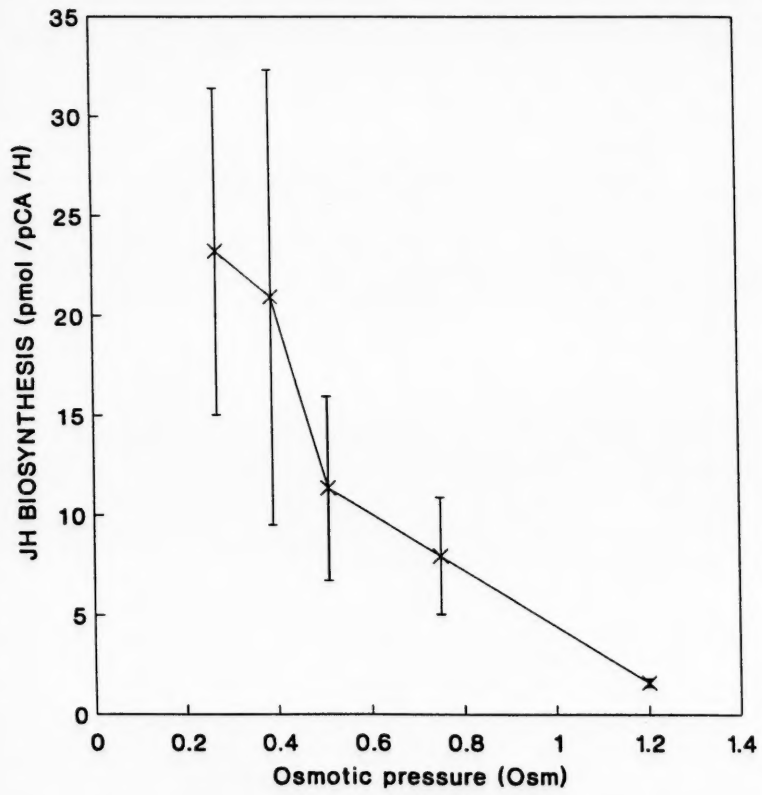

Fig. 4. Influence of changes in osmotic pressure of the incubation medium on the rate of JH biosynthesis. Changes in osmotic pressure were induced by NaCl addition in incubation medium. Each point (±SEM) represents the mean of ten incubations with single pairs of corpora allata from 8-day-old adult females.

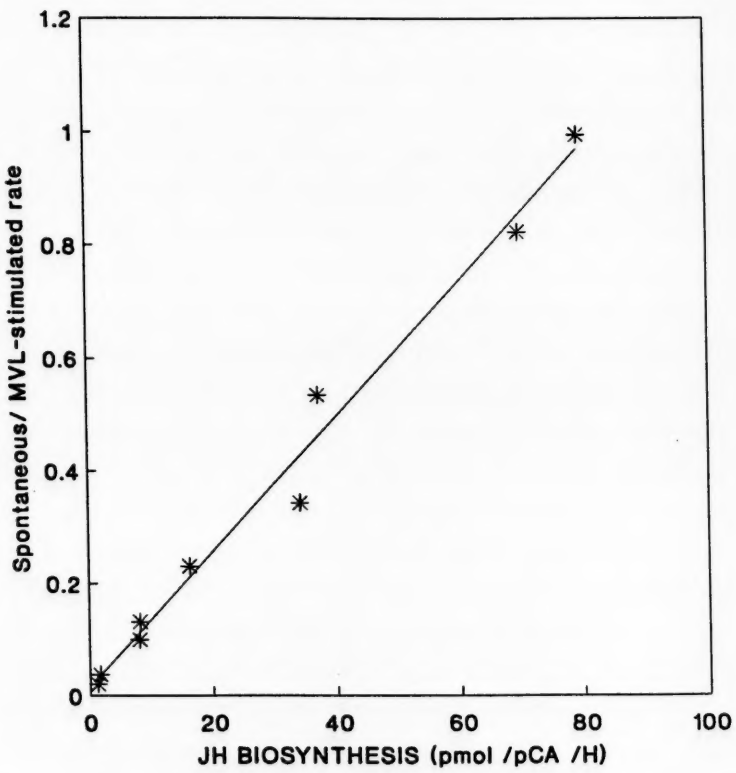

Fig. 6. Relation between the spontaneous rate and the ratio between spontaneous and 120 mM (3*RS*)-mevalonic acid lactone-stimulated rate of JH biosynthesis by individual pairs of corpora allata from 8-day-old adult females.

325 mOsm for respectively 50, 120 and 160 mM of racemic mevalonic acid lactone. Higher osmotic pressure was measured in mevalonate-supplemented medium with 308, 380 and 430 mOsm for respectively 50, 120 and 160 mM of racemic mevalonate. Fig. 4 illustrates the effect of osmotic pressure on the rate of JH biosynthesis. An increase in osmotic pressure resulted in a decline in the rate of JH biosynthesis. However, the higher osmotic pressure observed with mevalonate compared with mevalonic acid lactone cannot be the sole cause of the poor stimulating effect of mevalonate.

Mevalonate and mevalonic acid lactone on mevinolin-treated corpora allata

Because mevinolin is a potent inhibitor of HMG-CoA reductase and JH biosynthesis in locust corpora allata (B. Mauchamp and F. Couillaud, unpublished results), it was expected that mevinolin would suppress the level of endogenous mevalonate. Mevinolin (10 μM) suppressed JH production by corpora allata from 8-day-old adult females (Fig. 5). When mevalonic acid lactone was added to the incubation medium containing 10 μM mevinolin, JH production was restored at the mevalonic acid lactone-stimulated rate of JH biosynthesis, i.e. 2 times higher than the spontaneous rate (compare Fig. 5 and Fig. 1). Thus, the missing endogenous mevalonate is completely replaced by mevalonate from exogenous mevalonic acid lactone. However, mevalonate added in the incubation medium did not restore the spontaneous rate of JH biosynthesis in mevinolin-treated glands. Exogenous racemic mevalonate, even at 120 mM, did not replace the endogenous mevalonate (Fig. 5). The results suggested a poor penetration of mevalonate when compared to mevalonic acid lactone.

Ratio between spontaneous rate and mevalonic acid lactone-stimulated rate of JH biosynthesis

To characterize the stimulation of JH biosynthesis by exogenous mevalonic acid lactone, the ratio between spontaneous and mevalonic acid lactone-stimulated rate of JH biosynthesis (called FEAR, Tobe and Pratt, 1976) has been determined by successive 2 h incubations. For each individual pair of corpora allata, the ratio was

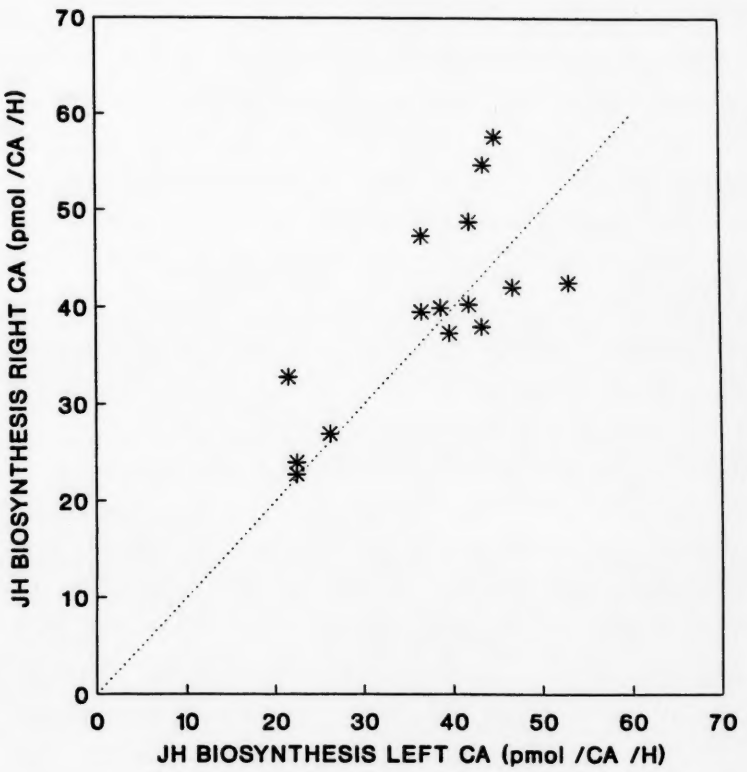

Fig. 7. Rate of JH biosynthesis by left and right corpora allata from 8-day-old adult females when stimulated with 120 mM (3*RS*)-mevalonolactone.

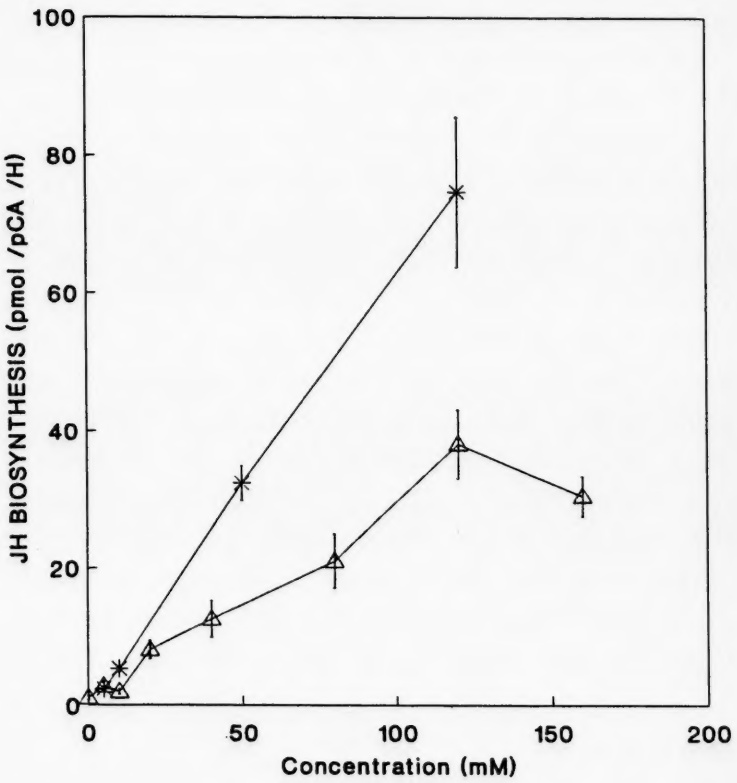

Fig. 8. Influence of mevalonate (△) and mevalonolactone (*) concentrations on the rate of JH biosynthesis by corpora allata from 11-day-old adult females with NCA-1 transected on day 8. Each point (±SEM) represents the mean of 6–10 incubations with single pairs of corpora allata.

always less than 1 (Fig. 6). Glands with low spontaneous activity exhibited low ratio values, while glands with high spontaneous activity exhibited high ratio values ($r = 0.99$; $n = 9$). This suggests that the spontaneous rate of JH synthesis did not reflect any differences in the capacity of the enzymatic steps posterior to mevalonate formation.

Asymmetry of JH biosynthesis and mevalonic acid lactone stimulation

Because left and right corpora allata in the locust present a large asymmetry in the spontaneous rate of JH biosynthesis and not in farnesoic acid or farnesol-stimulated rate (Couillaud and Girardie, 1987), we investigated asymmetry in mevalonic acid lactone-stimulated rate of JH production (Fig. 7). The mevalonic acid lactone-stimulated rate of JH biosynthesis was similar for the left and the right gland of a pair. Thus, the asymmetry in JH biosynthesis did not involve limiting activities of enzymes posterior to mevalonate formation.

Mevalonate and mevalonic acid lactone stimulation of disconnected corpora allata

Both mevalonate and mevalonic acid lactone were added in incubation medium in an attempt to restore JH biosynthesis by 11-day-old corpora allata that were isolated from the brain-corpora cardiaca complex by in situ transection of NCA-1 on day 8 (Fig. 8). Three days after the operation, racemic mevalonic acid lactone at 120 mM entirely restored the mevalonic acid lactone-stimulated rate of JH production typical of day-8 corpora allata. This suggests that enzymes acting after the mevalonate formation are still present in the corpora allata and thus are not responsible for the low rate of JH biosynthesis by disconnected corpora allata. Mevalonate also stimulated JH production by disconnected glands at a level close to the spontaneous rate of JH biosynthesis.

Discussion

The stimulation of JH-III biosynthesis by exogenous precursors such as farnesol and farnesoic acid gives useful insights into the regulation of the corpora allata because it is indicative of the degree of saturation of the enzyme situated beyond the entry of those precursors (Feyereisen, 1985; Schooley and Baker, 1985). Exogenous mevalonate has been shown to serve as JH-III precursor in the corpora allata of several insect species (see Schooley and Baker, 1985; Feyereisen and Farnsworth, 1987). However, the use of exogenous mevalonate as a probe of the physiology of the corpora allata has been restricted to the viviparous cockroach *Diploptera punctata*. Problems were encountered because of the limited penetration of mevalonate into the gland cells (Feyereisen and Farnsworth, 1987). For instance, mevalonate was not able to restore JH-III synthesis when endogenous mevalonate synthesis was blocked by the HMG-CoA reductase inhibitor mevinolin (Feyereisen and Farnsworth, 1987). Results presented in this paper largely support those conclusions as mevalonate was not able to restore JH synthesis in mevinolin-treated glands in the African locust.

However, mevalonolactone does restore JH synthesis in mevinolin-treated glands, and the mevalonolactone-stimulated rate of JH synthesis in mevinolin-treated glands is comparable to the mevalonolactone-stimulated rate of non-treated glands. In the locust, mevalonolactone stimulated the rate of JH biosynthesis by corpora allata of different ages (days 1, 5 and 8) and the stimulation appeared to be dependent on the spontaneous JH-III release rate. In *Locusta*, as in *Diploptera* (Feyereisen et al., 1981), low activity glands showed low ratio values (ratio between spontaneous and mevalonic acid lactone-stimulated rate of JH biosynthesis), whereas high activity glands exhibited high ratio values when stimulated with mevalonolactone. The present data show that the mevalonolactone-stimulated rate of JH biosynthesis always exceeded the spontaneous rate. Thus, under spontaneous synthesis conditions, enzymatic steps of JH biosynthesis subsequent to mevalonate formation are not rate limiting in corpora allata of adult female locusts.

Efficient concentrations for mevalonolactone stimulation of JH synthesis are very high and result in a progressive acidification of incubation medium. Decreasing the pH of the incubation medium reduces JH production by the corpora allata (Tobe and Pratt, 1974; this work Fig. 3). These harmful circumstances of incubation may

be responsible for the relatively low mevalonolactone-stimulated rates of JH synthesis (60 pmol/pair of corpora allata/h) when compared with farnesol or farnesoic acid-stimulated rate (around 250 pmol/pCA/h) (Couillaud et al., 1988). The slight stimulation may be also attributable to cell membrane permeability problems (Feyereisen et al., 1981). Thus, the activities measured in the presence of mevalonolactone are lower than the maximal velocities of enzymatic steps posterior to mevalonate formation (at least for the alcohol and aldehyde dehydrogenase, methyl transferase and epoxidase) and therefore probably do not reflect the total amount of enzyme present in the corpora allata. At least, the slight stimulation may indicate that the concentration of enzymes involved in synthesis from mevalonate to farnesol is lower than the concentration of enzymes converting farnesol into JH-III.

Mevalonolactone has been used to investigate the asymmetry in spontaneous rate of JH biosynthesis by locust corpora allata. When stimulated by mevalonolactone, left and right glands exhibited very similar rates of JH biosynthesis as had been previously shown with farnesoic acid (Couillaud and Girardie, 1987). Thus, enzymatic equipment for JH biosynthesis from mevalonate does not differ between left and right glands and asymmetry in spontaneous rate of JH biosynthesis may involve some kind of individual regulation of each gland acting prior to mevalonate. Morphological and experimental evidence suggest that this individual regulation comes from allatostimulating factors of the lateral neurosecretory cells (Couillaud and Girardie, 1987). Some tracts of the lateral neurosecretory cells extend to the corpora cardiaca, cross them and enter the NCA-1 to reach the corpora allata (Cassier and Fain-Maurel, 1970). This organisation provides opportunities for local action directly on allatal cells. Furthermore, deprivation of lateral factors by destruction of the lateral neurosecretory cells, or NCA-1 transection result in a decline of JH biosynthesis (no stimulation) (Couillaud and Girardie, 1985). Disconnected corpora allata exhibit then a constant low rate of JH biosynthesis. Severance of the left NCA-1 results in a decrease in the rate of JH biosynthesis only by the disconnected gland, suggesting that the stimulating factor of the right size cannot act on the left size through the hemolymph (Couillaud and Girardie, 1987).

We show in this paper that mevalonolactone is able to restore JH biosynthesis in disconnected corpora allata (NCA-1 transection 3 days before). Enzymatic steps subsequent to mevalonate formation are not responsible for the low rate of JH biosynthesis by disconnected glands and thus, the allatostimulating factor coming through the NCA-1 acts on JH biosynthesis prior to mevalonate. Three days after the NCA-1 transection, enzymes subsequent to mevalonate formation are still present. This is consistent with the fact that brain extract can stimulate JH biosynthesis by disconnected corpora allata during the 3 h time of an in vitro incubation (Couillaud and Girardie, 1990) and supports the idea of 'a rate limiting step' prior to mevalonate kinase (i.e. acetoacetyl-CoA thiolase, HMG-CoA synthase and HMG-CoA reductase).

Whether the allatostimulating factor coming through the NCA-1 is also responsible for the asymmetry in spontaneous rate of JH biosynthesis remains to be clearly established but the present data show that both asymmetry and stimulation by the NCA-1 factor involve early steps in the JH pathway prior to mevalonate.

Acknowledgments

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Effects of activin A on anterior pituitary cells fractionated by centrifugal elutriation

Tetsuro Katayama, Kunio Shiota and Michio Takahashi

Department of Veterinary Physiology, Veterinary Medical Science, The University of Tokyo, 1-1-1 Yayoi, Bunkyo-ku, Tokyo 113, Japan

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Key words: Activin A; Anterior pituitary; Centrifugal elutriation; Follicle-stimulating hormone

Summary

We have shown previously that activin A increases the number of immunoreactive follicle-stimulating hormone (FSH) cells. To further investigate the action of activin A, we examined its effects on anterior pituitary cells fractionated by centrifugal elutriation. Before activin A treatment, FSH cells were widely distributed among various fractions; a higher proportion of FSH cells was found in larger cell fractions (fractions 5–9), and a lower proportion in smaller cell fractions (fractions 2–4). After culture of the cells in each fraction with activin A (10 ng/ml) for 72 h, the number of FSH cells in fraction 4 only was significantly ($P < 0.05$) higher by 225% than that in cells cultured without activin A. The amount of FSH secreted into the medium was minimal or undetectable in fractions 1–4. However, FSH secretion tended to be, or was significantly ($P < 0.01$ in fraction 9), stimulated by activin A in fractions 5–9, in which the numbers of FSH cells were not significantly affected.

These results suggest a dual mode of action of activin A on FSH: activin A increases the number of FSH cells in a specific type(s) of middle-sized cell fraction, and stimulates FSH secretion at least from larger cells without affecting the number of FSH cells.

Introduction

Activins (A, AB) are glycoproteins which were originally purified from ovarian fluid as stimulators of follicle-stimulating hormone (FSH) secretion in anterior pituitary cells (Ling et al., 1986a, b; Vale et al., 1986). Activin A has been shown to be identical to erythroid differentiation factor (EDF) isolated from a human monocytic leukemia cell line as a stimulator of differentiation of Friend erythroleukemia cells (Eto et al., 1987; Murata et al., 1988).

We have shown that activin A increases the number of immunoreactive FSH cells in vitro (Katayama et al., 1990), suggesting that the agent acts as a proliferation- or differentiation-promoting factor on anterior pituitary cells. Thus, with regard to the mode of action of activin A on anterior pituitary cells, it has been deduced that

Address for correspondence: Tetsuro Katayama, D.V.M., Department of Veterinary Physiology, Veterinary Medical Science, The University of Tokyo, 1-1-1 Yayoi, Bunkyo-ku, Tokyo 113, Japan.

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some type(s) of cell other than FSH cells may be the main target of activin A, and that the effect of activin A cannot solely be expressed through a quantitative change in such cells. Since an immunocytochemical study has shown that the total number of gonadotropes is increased, but the proportions of luteinizing hormone, thyroid-stimulating hormone, prolactin (PRL) and growth hormone cells are unchanged by activin A treatment (Katayama et al., 1990), the increase in the number of FSH cells is not due to conversion of other endocrine cell types into FSH cells. It is of considerable interest which cell type(s) is the target for the action of activin A.

Another question is raised by the finding that the rates of increase in the number of FSH cells and in FSH secretion were not equivalent upon stimulation with activin A (Katayama et al., 1990): although FSH secretion was stimulated about 2-fold by activin A, the increases in the number of FSH cells were restricted to about 1.4-fold. Thus, it is likely that another mode of action is also involved in the effect of activin A.

In this study, in order to further investigate the mode of action of activin A on FSH secretion, anterior pituitary cells were fractionated according to cell size by centrifugal elutriation, and the effects of activin A on the number of FSH cells and on FSH secretion were examined in each fraction separately.

Materials and methods

Animals

Male Wistar-Imamichi rats (10–15 weeks old) obtained from the Imamichi Institute for Animal Reproduction (Ohmiya, Japan) were maintained for at least one week in a controlled environment at $23 \pm 1°C$ with a 14 h light:10 h dark cycle (lights on at 05:00 h). The animals had free access to water and standard laboratory chow.

Reagents

Activin A (EDF), isolated from a human monocytic leukemia cell line THP-1 (Tsuji et al., 1988), was a gift from the Central Research Laboratory of Ajinomoto Co. (Kawasaki, Japan). Other materials were obtained from the following sources: Dulbecco's modified Eagle medium (DMEM), fetal calf serum (FCS), deoxyribonuclease (DNase) and pancreatin from Grand Island Biological Co. (Grand Island, NY, U.S.A.); *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (Hepes), bovine serum albumin (BSA) (fatty acid-free), penicillin and streptomycin sulfate from Sigma Chemical Co. (St. Louis, MO, U.S.A.); collagenase (type II) from Worthington Biochemical Co. (Freehold, NJ, U.S.A.).

Cell dispersion

Anterior pituitary glands from 25 rats were minced and washed with Hepes buffer (137 mM NaCl, 5 mM KCl, 0.7 mM Na_2HPO_4 and 25 mM Hepes, pH 7.3), and cell dispersion was performed by the method reported previously (Shiota et al., 1984; Katayama et al., 1990). Briefly, the tissue was dispersed by incubation first in Hepes buffer containing 0.4% collagenase, 0.4% BSA, 10 μg/ml DNase and 0.2% glucose, and then with 10% pancreatin. Average cell viability in this series of studies determined by trypan blue exclusion was more than 90%. The cells were suspended in 5 ml of DMEM and the cell number was counted.

Elutriation

The standard separation chamber of an SRR6Y rotor system (Hitachi Koki Co., Katsuta, Japan) and all tubing were sterilized with 70% ethanol, and rinsed with sterile distilled water and Hepes buffer. The rotor speed was held constant at 1960 rpm ($380 \times g$) throughout the fractionation process. Cells ($1.8–2.2 \times 10^7$) were pumped into the chamber (22° C) at a flow rate of 8.5 ml/min, and were elutriated in 100 ml volumes of Hepes buffer by increasing the flow rate from 14 to 47 ml/min (i.e., 14, 18, 22, 27, 32, 37, 42 and 47 ml/min). After the final (8th) fraction had been collected, the rotor was stopped, and cells remaining in the chamber were collected in 100 ml of Hepes buffer as the wash fraction (fraction 9). After centrifugation at $480 \times g$ for 6 min, the supernate was aspirated off and the cells of each fraction were resuspended in 3 ml of DMEM supplemented with 10% FCS. An aliquot of each fraction was utilized for counting the cell number or for determining the viability of the cells by trypan blue exclusion.

Culture conditions and activin A treatment

The cells of each fraction were adjusted to a concentration of 1×10^5 cells/ml with DMEM containing 10% FCS, and 160 μl or 1 ml of the cell suspension was placed in each well of sterile 96-well or 24-well culture dishes, respectively (Costar, Cambridge, MA, U.S.A.). The cells were incubated under a saturated atmosphere of 5% CO_2–95% air in a CO_2 incubator at 37°C. Then, after 18 h, the medium was exchanged for a fresh one containing 10% FCS with or without activin A (10 ng/ml) and the cells were further incubated for 72 h.

Immunocytochemistry

After washing with DMEM without FCS, the cells were fixed in Bouin's fluid (without acetic acid) for 10 min at room temperature and then stained immunocytochemically using peroxidase-antiperoxidase (PAP) reagents (BIO GENEX Laboratories, CA, U.S.A.). The optimum dilutions of the primary antibodies were 1 : 1000 for NIDDK-anti-rFSH-S-11 and 1 : 10,000 for NIADDK-anti-rPRL-IC-3. The immunostaining was carried out as reported elsewhere (Katayama et al., 1990). Briefly, the sequences and reaction times were as follows: 3% hydrogen peroxidase for 10 min; normal goat serum for 5 min; primary antibody (not supplied in the kit) for 1 h; goat anti-rabbit IgG for 5 min; PAP complex for 5 min; peroxidase substrate (3-amino-9-ethyl-carbazole) for 10 min. All the steps were run at 37°C. In the experiment to evaluate the specificity of the staining, normal rabbit serum or anti-FSH serum preabsorbed with FSH was used instead of the primary antibody. More than 200 cells from five independent areas in each of two wells were counted using a light microscope, and visibly stained cells were considered to be immunoreactive cells.

Measurement of secreted FSH levels

At the end of the incubation, the collected medium was centrifuged ($480 \times g$ for 6 min) and the supernate was stored at −20°C until assay of FSH levels. The amount of FSH was measured in duplicate by double-antibody radioimmunoassay using a kit provided by the NIADDK. The data were expressed in terms of NIADDK-rat FSH-RP-2.

Statistics

Percentages of immunoreactive cells were presented as mean ± SEM for each experimental group, and the percentages of immunoreactive FSH cells in activin A-treated groups as rates of increase versus each control mean value. Significance of the difference of values between activin A-treated and untreated cells was evaluated by Student's *t*-test.

Results

Cell recovery after elutriation

Cell numbers in elutriated fractions based on cell counts are shown in Fig. 1. The number of elutriated cells increased gradually with increasing fraction number, and reached a maximum in fraction 5. Then, the number began to decrease and reached a minimum in fraction 8. Fig. 2 shows the cells in fractions 2, 4 and 8. It is evident that cells were separated into the different fractions according to size, and that the cell size in each fraction was almost uniform. The cells in the wash fraction (fraction 9) were not as uniform in size as those in fractions 1–8 (data not shown). The average viability of the elutriated cells was 87.5%, which was similar to that of the initial cell suspension before

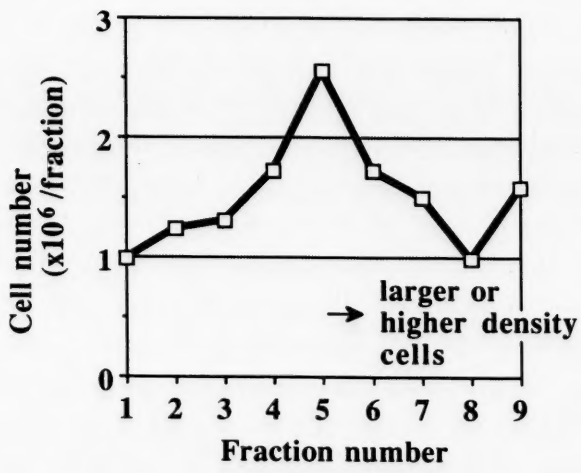

Fig. 1. Cell numbers in the elutriated fractions. The rotor speed was held constant at 1960 rpm ($380 \times g$) and fractions 1–8, each 100 ml, were collected at flow rates of 14, 18, 22, 27, 32, 37, 42 and 47 ml/min, respectively. Fraction 9 was collected as the 100 ml fraction after the rotor had stopped.

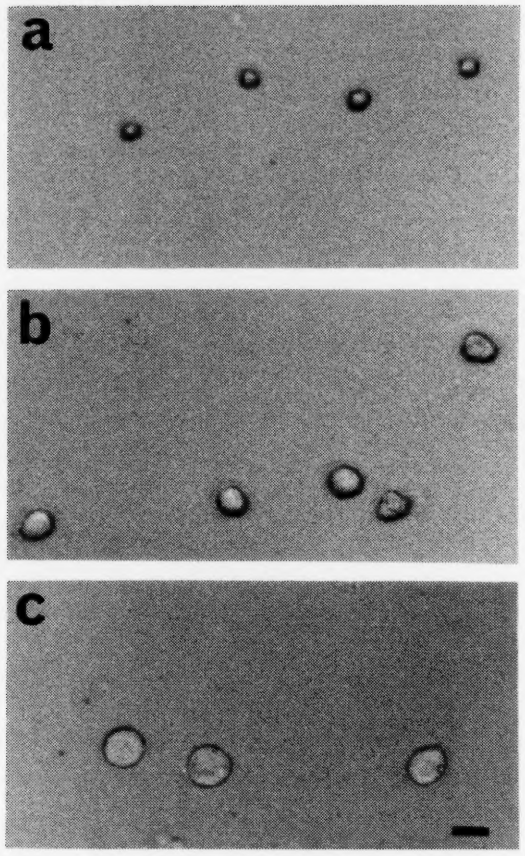

Fig. 2. Light micrographs of cells in fraction 2 (*a*), fraction 4 (*b*) and fraction 8 (*c*). The cells were fixed in Bouin's fluid 18 h after being plated on culture dishes, and then photographed. Magnifications are identical in (*a*), (*b*) and (*c*). Bar = 15 μm.

elutriation (91.7%), indicating that damage to cells by the elutriation procedure was minimal.

Distribution of immunostained cells after elutriation

Fig. 3 shows the percentages of FSH and PRL cells in the initial cell suspension and in the elutriated fractions. Cells were fixed and stained 18 h after being plated on culture dishes, before the initiation of activin A treatment. Cells staining for FSH accounted for 6.15 ± 0.26% of the initial cell suspension. After elutriation, FSH cells were widely distributed in all fractions except for fraction 1. When compared with the value in the initial cell suspension, the percentage of FSH cells was relatively high in fractions 5–9, and highest in fraction 8. In contrast, fractions 2–4 included fewer FSH cells. Cells staining for PRL accounted for 29.8 ± 1.1% of the initial cell suspension, and a higher proportion of these cells was found in fractions 2 and 3. In this series of studies, no staining was observed when normal rabbit serum or anti-FSH serum preabsorbed with FSH was used. Total numbers of elutriated FSH cells and PRL cells corresponded to 6.72% and 30.3% of the total number of elutriated cells, respectively, indicating that the elutriation procedure did not cause loss of any specific cell types.

Effects of activin A on the number of FSH cells in elutriated fractions

The cells of each fraction were cultured with or without activin A (10 ng/ml) for 72 h, and the proportions of immunoreactive FSH cells were compared between activin A-treated cells and untreated control cells. The percentage of FSH cells in activin A-treated cells was not significantly different from that in control cells in fractions 5–9. In the fractions containing smaller cells, no significant difference in the proportion of FSH

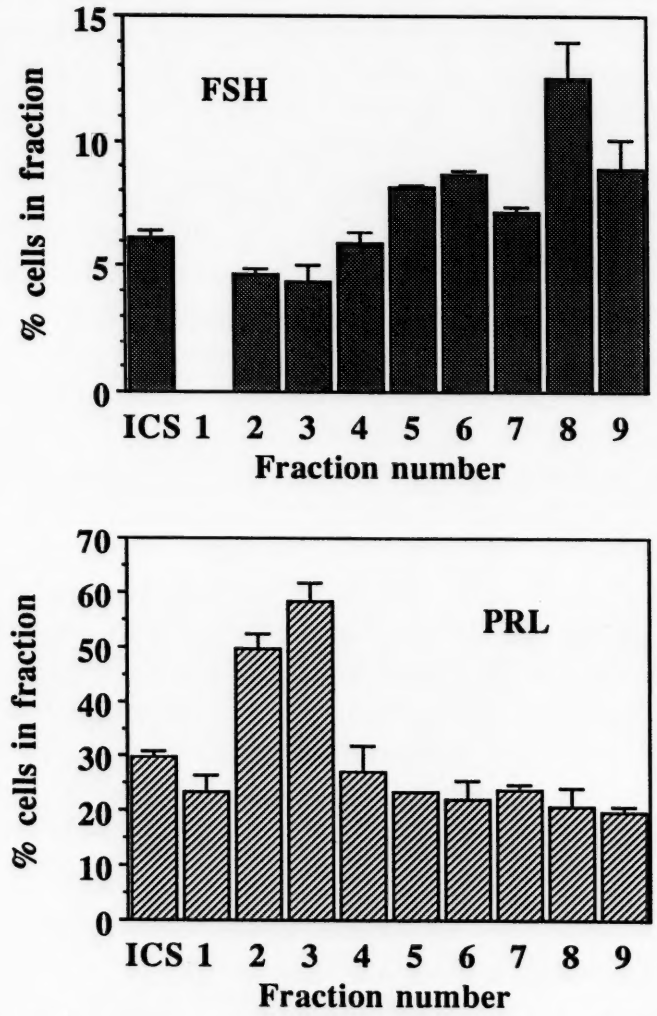

Fig. 3. Percentages of FSH- and PRL-containing cells in the initial cell suspension (ICS) and in the elutriated fractions. The cells were fixed and stained before the initiation of activin A treatment. Immunocytochemically stained cells were counted at the light microscopic level, and the values are presented as means ± SEM for two trials.

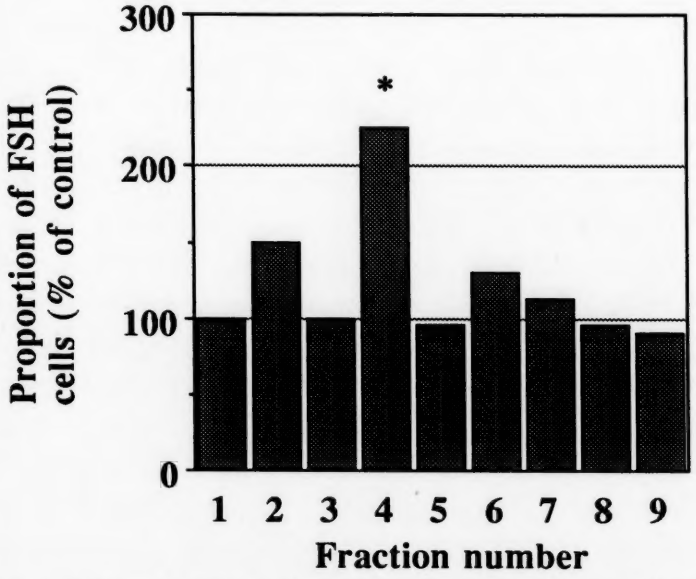

Fig. 4. Effects of activin A on the number of FSH cells in the elutriated fractions. The cells in each fraction were precultured for 18 h, and then cultured in fresh medium with or without activin A (10 ng/ml) for 72 h. Immunocytochemically stained cells were counted at the light microscopic level, and the percentages of immunoreactive FSH cells in activin A-treated groups are presented as rates of increase versus each control value. * Difference of values between activin A-treated and untreated cells is significant ($P < 0.05$, $n = 2$).

cells between activin A-treated and control cells was observed in fractions 1–3. However, the percentage of FSH cells among activin A-treated cells in fraction 4 was specifically and significantly ($P < 0.05$) higher (2.25-fold) than that in control cells (Fig. 4). These results showed that the stimulatory effect of activin A on the number of FSH cells was not expressed randomly on anterior pituitary cells, but specifically on middle-size cells in fraction 4.

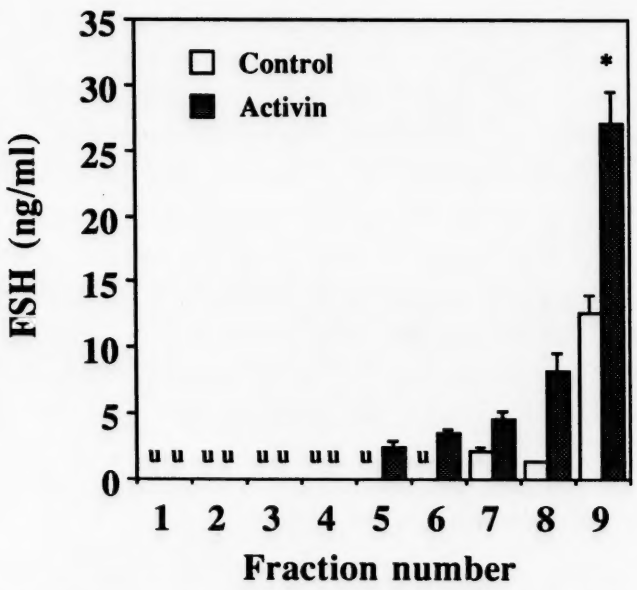

Fig. 5. Effects of activin A on FSH secretion in the elutriated fractions. The cells in each fraction were precultured for 18 h, and then cultured in fresh medium with or without activin A (10 ng/ml) for 72 h. Amounts of immunoreactive FSH secreted into the medium during a 72 h incubation were assayed. The values are expressed as means ± SEM for three trials. * Difference of values between activin A-treated and untreated cells is significant ($P < 0.01$); u, undetectable level (< 1.25 ng/ml).

Effects of activin A on FSH secretion in elutriated fractions

Amounts of immunoreactive FSH secreted into the medium during the 72 h incubation with or without activin A were measured in each fraction separately. In fractions 1–4, FSH levels were very low and were detectable neither in control nor in activin A-treated cells. The levels were calculated to be below 1.25 ng/ml. In fractions 5 and 6, amounts of FSH were not yet measurable in control cells, whereas in activin A-treated cells FSH levels were high enough to be detected. In the fractions containing larger cells, fractions 7–9, amounts of FSH could be detected in both groups. The level of FSH increased gradually with increasing fraction number from 5 to 9 in activin A-treated cells, and a similar tendency was observed in control cells of fractions 7–9. In these fractions, the amounts of FSH released into the medium were apparently higher in activin A-treated cells than in control cells, and the difference was statistically significant ($P < 0.01$) in fraction 9 (Fig. 5). From these results, it was indicated that FSH release was higher in larger cell fractions than in smaller ones, and that activin A could stimulate FSH secretion at least from larger types of cells on which the effect of activin A was not expressed in terms of FSH cell number (Fig. 4).

Discussion

The results of the present study confirmed that centrifugal elutriation was effective for fractionating anterior pituitary cells according to cell size (Hyde et al., 1982). This fractionation method yields specific cell type-enriched populations with minimal cell damage within 1 h. Moreover, since each type of anterior pituitary cell, i.e., gonadotrope or lactotrope, is heterogeneous in shape and size (Nakane, 1970), the method is also useful for

fractionating one cell type into several subtypes, as this study confirmed.

Our present findings confirmed those obtained previously that an increase in the number of FSH cells was involved in the mechanism of action of activin A on anterior pituitary cells (Katayama et al., 1990). In this study, we found that the stimulatory effect of activin A, inducing cells to represent a higher number of FSH cells than that without activin A treatment, was manifested in a specific cell fraction following centrifugal elutriation. This means that this mode of action of activin A was not expressed randomly on cells distributed widely throughout all fractions, but on a type of cell with both uniform size and specific gravity.

Levels of FSH secreted into the medium were much higher in larger cell fractions (fractions 5–9) than in smaller ones (fractions 1–4). For instance, the differences between fraction 9 and fractions 1–4 were calculated to be at least 10-fold, respectively. Since the number of FSH cells in each culture ranged from 4.4 to 12.5×10^3 cells/well, the obvious difference in the amounts of secreted FSH between larger and smaller cells was thought to depend on a difference in the FSH-secreting activity. This would support the concept that larger types of FSH cells are at higher stages of differentiation than smaller types (Naor et al., 1982; Lloyd and Childs, 1988).

Our results showed that activin A did not manifest its effect on cells in the fractions containing larger cells. This suggests that FSH cells at higher stages of differentiation may not be responsive to activin A. In other words, since these larger types of FSH cells constituted the majority of FSH cells before the initiation of 72 h culture, the main population of FSH cells is not responsive to activin A. These results support the concept that the increase in the number of FSH cells in activin A-treated cells compared with that in untreated cells might not be attributable to the proliferation of well-differentiated FSH cells.

With regard to the increase in the cell number, the cells responsive to activin A were smaller in size than the main populations of FSH cells which were more highly differentiated, suggesting that these smaller or middle-sized FSH cells at a lower stage of differentiation might be those which proliferate in response to activin A. Recently, Schubert et al. (1990) have reported that activin A promotes the survival of some kinds of less differentiated cells, such as P19 teratoma cells or rat embryonic neural retina cells. Accordingly, it might also be assumed that less differentiated FSH cells in fraction 4 were rescued from degeneration by activin A, resulting in a higher number of FSH cells remaining among activin A-treated cells than among untreated cells after 72 h culture. Alternatively, it is possible that some as yet undefined, hormonally negative cells responded to activin A, since the cells responsive to activin A were present in the fraction which contained fewer FSH cells before the start of activin A treatment. In this case, either proliferation or differentiation of such undefined, undifferentiated cells might be involved.

Another mode of action of activin A was found by examining its effects on FSH secretion. In the fractions containing larger cells, FSH secretion was stimulated by 72 h incubation with activin A, although the numbers of immunoreactive FSH cells were not affected. These findings indicate that a stimulatory effect on FSH release from individual FSH cells of the larger type was also involved in the action of activin A. This mode of action might explain why the rate of increase in the number of immunoreactive FSH cells does not parallel the rate of FSH secretion from whole anterior pituitary cells (Katayama et al., 1990).

In summary, we have shown that (1) activin A increases the number of FSH cells in the middle-sized cell fraction, its action thus being directed at a specific cell fraction, and that (2) activin A also stimulates FSH secretion at least from larger cells without affecting the number of FSH cells. It is likely that a dual mode of action is involved in the effect of activin A on FSH secretion.

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Insulin-like growth factor-I gene expression during development and estrous cycle in the rat uterus

Björn Carlsson and Håkan Billig

Department of Physiology, University of Göteborg, S-400 33 Göteborg, Sweden

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Key words: Insulin-like growth factor-I mRNA; Estradiol; (Rat uterus)

Summary

The ontogeny and estrous cycle-dependent variation of insulin-like growth factor-I (IGF-I) gene expression was analyzed in the rat uterus. RNA extracted from rat uteri contained transcripts with estimated sizes of 7.0, 1.7, and 1.2–0.8 kb that were recognized by a ^{32}P-labelled mouse IGF-I RNA probe. A solution hybridization RNase protection assay was used to measure the abundance of IGF-I mRNAs in uteri from rats of different ages. The highest levels were found in adult rats ($p < 0.01$). The levels of IGF-I transcripts changed markedly during the estrous cycle with the highest levels at proestrus ($p < 0.01$). There was an 8-fold increase in the abundance of IGF-I mRNA between diestrus-2 and proestrus. The corresponding livers had no significant variation of IGF-I gene expression during the estrous cycle, demonstrating a tissue-specific regulation of the IGF-I gene. The time and dose dependency of estrogen regulation of IGF-I gene expression was studied in hypophysectomized rats. The levels of IGF-I mRNA in the uterus decreased after hypophysectomy. A single s.c. injection of estradiol significantly increased the levels of IGF-I transcripts after 3 h ($p < 0.01$). A low dose of estradiol (0.1 μg/100 g) increased the levels of IGF-I transcripts but progesterone in higher doses (5 μg/100 g) was without effect, indicating that the effect was specific for estradiol. However, the present study provides no information regarding whether this regulation is at the level of transcription or mRNA stability.

The present study provides further support for estrogen as a major regulator of IGF-I gene expression in the uterus in vivo.

Introduction

Estrogen is a major stimulator of uterine growth in vivo. The proliferative effects have been difficult to achieve on cultured uterine epithelial, myometrial, and endometrial cells (Chen et al., 1973; Gerschenson et al., 1977; Pavlic et al., 1978; Tomooka et al., 1986). This has led to the suggestion that the effects of estrogen are mediated by other factors. Murphy et al. have suggested that insulin-like growth factor-I (IGF-I) is an autocrine and paracrine mediator of estrogen action in the uterus. This is supported by the demonstration of local production of IGF-I in the uterus (Murphy et al., 1988), the presence of IGF-I receptors (Ghahary and Murphy, 1989), the uterus having the highest extrahepatic steady-state levels of

Address for correspondence: Dr. Björn Carlsson, Department of Physiology, University of Göteborg, P.O. Box 33031, S-400 33 Göteborg, Sweden.

IGF-I gene expression (Murphy et al., 1987), and also that IGF-I potentiates estrogen-stimulated DNA synthesis in cultured uterine sections (Ghahary et al., 1990). Growth hormone (GH) is the major regulator of IGF-I gene expression in the liver and many extrahepatic tissues (Mathews et al., 1986). In contrast, estradiol is more potent than GH in increasing the levels of IGF-I transcripts in the uterus (Murphy and Friesen, 1988). Most studies so far have focused on the regulation of IGF-I gene expression in immature or hypophysectomized rats.

In the present study we investigated the steady-state levels of IGF-I gene expression in the uterus of intact rats of different ages and during the estrous cycle. Furthermore, we used hypophysectomized rats to study the time course and dose dependency of estrogen regulation of the levels of IGF-I transcripts.

Materials and methods

Animals

Female Sprague-Dawley rats (Alab, Stockholm, Sweden) were housed under standardized environmental conditions with constant temperature (24–26 °C), humidity (50–60%) and artificial 14 h light:10 h dark cycle. The animals had free access to water and pelleted food. The rats were delivered at least 2 days before the start of the experiment. Immature (28-day-old) rats were hypophysectomized by a standard parapharyngeal approach. In the studies where the expression of the IGF-I gene was analyzed at specific days of the estrous cycle only rats exhibiting at least two consecutive 4-day cycles, as determined by daily vaginal smears, were used. The animals were killed by cervical dislocation and the tissues were immediately removed, trimmed with microscissors and rapidly frozen in liquid nitrogen. All tissues were stored at −70 °C until analyzed.

Probe

A 153 basepaire (bp) *Sma*I fragment of a genomic subclone of mouse IGF-I from exon 3 (by analogy to human IGF-I) subcloned into pSP64 (Mathews et al., 1986) was used as a template for probe synthesis. The plasmid was linearized with *Eco*RI and used as template for production of IGF-I (^{32}P, ^{35}S)-UTP-labelled RNA according to the manufacturer with SP6 RNA polymerase (Promega, Madison, WI, U.S.A.).

Northern blot analysis

Total RNA was prepared essentially according to Chomczynski and Sacchi (1987) with minor modifications (Nilsson et al., 1990). 20 μg of total RNA was electrophoresed in an agarose (1%) formaldehyde (2.2 M) gel with ethidium bromide. The RNA was transferred to Hybond-N membranes (Amersham, Buckinghamshire, U.K.) with a vacuum transfer system (LKB, Stockholm, Sweden). The membranes were baked at 80 °C for 3 h, prehybridized at 57 °C overnight in 50% formamide, 25 mM H_2NaPO_4, 25 mM HNa_2PO_4, 5 × saline sodium citrate (SSC), 0.1% sodium dodecyl sulfate (SDS), 1 mM EDTA, 0.05% bovine serum albumin (BSA), 0.05% Ficoll, 0.05% polyvinyl-pyrrolidone (PVP), 200 μg/ml calf liver RNA, and 200 μg/ml salmon sperm DNA, and hybridized in the prehybridization buffer with the addition of ^{32}P-labelled IGF-I RNA probe. The membranes were washed in 0.1 × SSC, 0.1% SDS at 65 °C, treated with RNase A (1 μg/ml) at 37 °C, 30 min, and washed in 0.1 × SSC, 0.1% SDS at 37 °C. A 0.24–9.5 kb RNA ladder (BRL, MD, U.S.A.) was used to estimate transcript sizes. Autoradiography was done using Kodak XAR-5 film (Eastman Kodak Co., Rochester, NY, U.S.A.).

Solution hybridization

Frozen tissue was homogenized with a polytron in 1% SDS, 20 mM Tris-HCl (pH 7.5) and 4 mM EDTA. The homogenate was treated with proteinase-K and the total nucleic acids (TNA) were extracted with phenol-chloroform (Durnam and Palmiter, 1983). A solution hybridization was used to quantify IGF-I mRNA as described elsewhere (Mathews et al., 1987). In brief, the hybridization was done to TNA samples at 70 °C for 24 h in 0.06 M NaCl, 20 mM Tris-HCl (pH 7.5), 4 mM EDTA, 0.1% SDS, 10 mM dithiothreitol (DTT), 25% formamide, and ^{35}S-labelled IGF-I RNA probe. After the addition of 100 μg herring sperm DNA the samples were treated with 40 μg/ml RNase A and 2 μg/ml RNase T_1 (Sigma, St. Louis, MO, U.S.A.). Trichloroacetic acid-precipitated protected probe was collected on glass-fiber

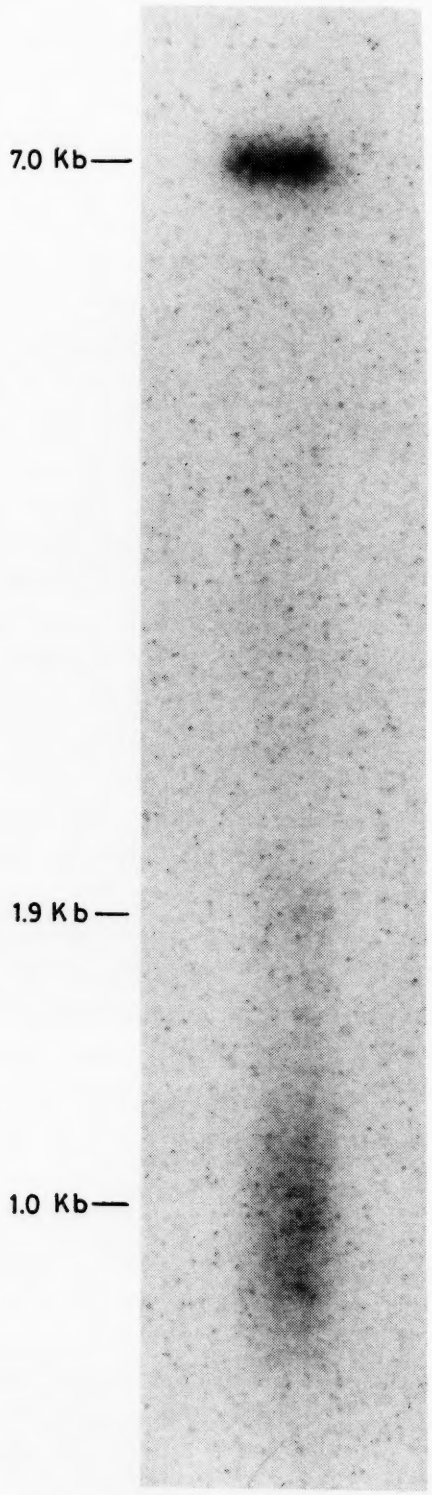

Fig. 1. Analysis of IGF-I gene expression in the uterus from an adult rat. Total RNA was electrophoresed, transferred and hybridized with a ^{32}P-labelled mouse IGF-I RNA probe under the conditions described in Materials and Methods.

filters (GF/C Whatman, Whatman International, Maidstone, U.K.) and counted in a scintillation counter. The signal was then compared to a standard curve based on hybridization to known amounts of IGF-I RNA. The DNA content in the TNA preparation was analyzed (Labarca, 1980) and the results were expressed as amol IGF-I RNA/μg DNA.

Statistical analysis

Values are given as mean $\pm$ SEM. The statistical differences between groups were analyzed by one-way analysis of variance followed by the Student-Newman-Keul's multiple test.

Results

RNA extracted from uteri was analyzed on a Northern blot with a mouse IGF-I RNA probe. Three major bands were detected with estimated sizes of 7.0, 1.7 and 1.2–0.8 kb (Fig. 1). The probe was further used in a solution hybridization RNase protection assay to determine the abundance of IGF-I transcripts in the uteri during different experimental conditions, however, this method provides no information if changes in mRNA levels are due to altered rate of transcription or mRNA stability. To study the ontogeny of IGF-I gene expression in the uteri, rats were killed at different ages and the concentration of IGF-I transcripts was determined. The highest levels were found in uteri of adult rats (Fig. 2). Adult rats were monitored by daily vaginal smears and rats with at least two consecutive 4-day cycles were used to analyze the abundance of IGF-I tran-

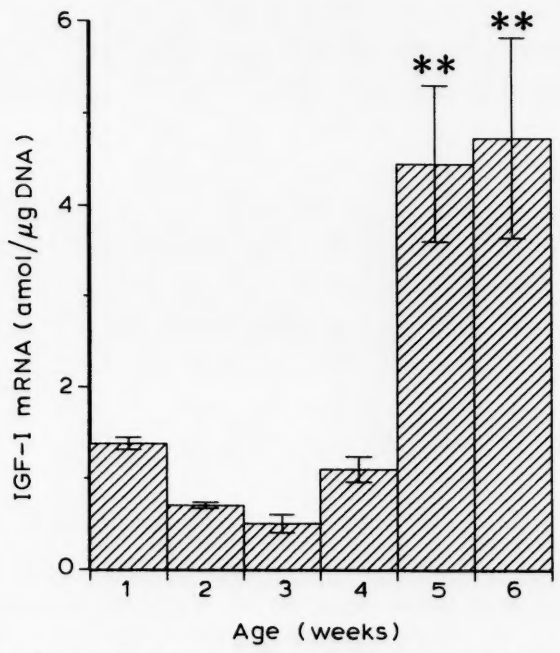

Fig. 2. Concentrations of IGF-I transcripts in the uterus at different ages in the rat. The level of IGF-I mRNA was determined with a solution RNase protection assay. Values are mean $\pm$ SEM. There were 5–8 rats in each group.

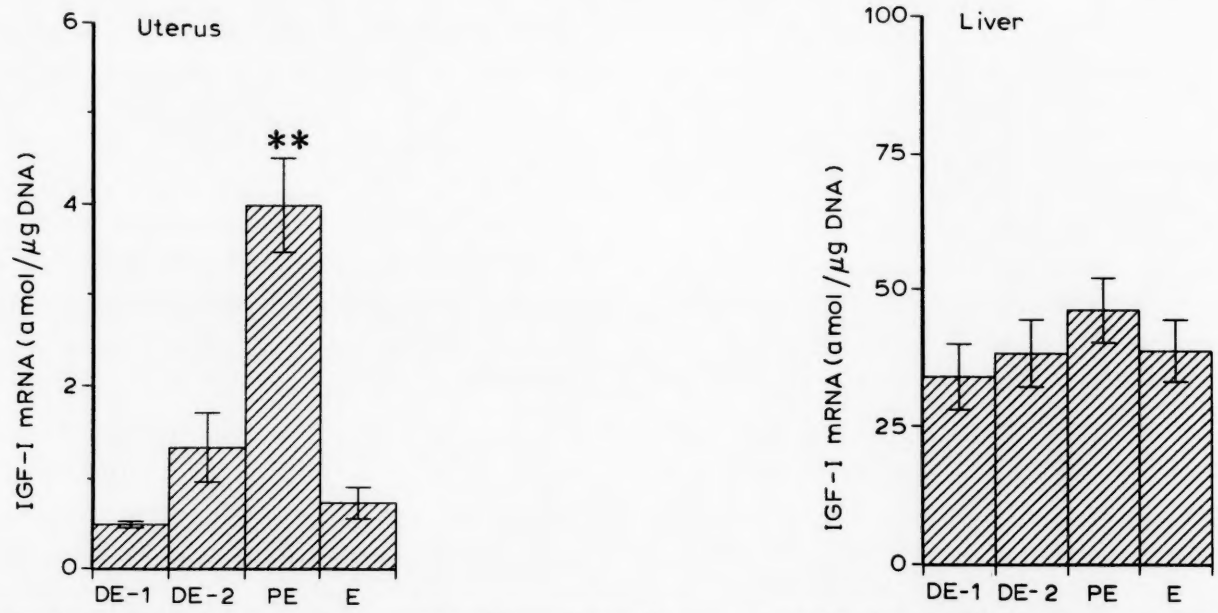

Fig. 3. Concentrations of IGF-I transcripts in uterus and liver during the estrous cycle in the rat (DE-1 = diestrus-1, DE-2 = diestrus-2, PE = proestrus, E = estrus). The rats, representing each day of the estrous cycle, were sacrificed at 12.00 h. The level of IGF-I mRNA was determined with a solution RNase protection assay. Values are mean ± SEM. There were 4–5 rats, individually analyzed, in each group.

scripts in the uterus at different days of the estrous cycle. As shown in Fig. 3, the highest concentration of IGF-I transcripts was present at proestrus.

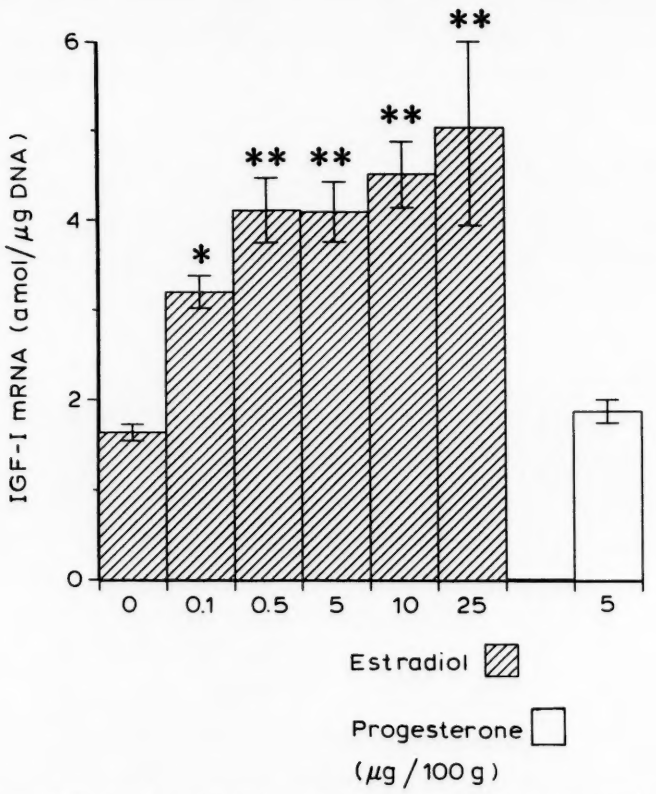

Fig. 4. Effect of estradiol on the level of IGF-I transcripts in the uterus of hypophysectomized rats. The animals were sacrificed 6 h after a single s.c. injection of estradiol. The level of IGF-I mRNA was determined with a solution RNase protection assay. Values are mean ± SEM. There were five rats, individually analyzed, in each group.

Since the pattern of IGF-I gene expression resembled that of estradiol during the estrous cycle, the time and dose dependency of estradiol regulation of IGF-I were examined. 28-day-old hypophysectomized rats were given a single s.c. injection of estradiol (E_2) and sacrificed after 6 h. The levels of IGF-I transcripts were dose-dependently regulated by estradiol, with a significant

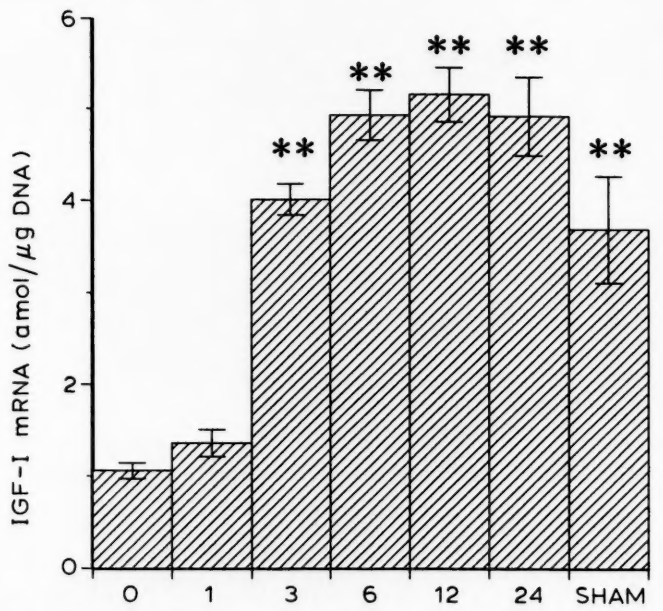

Fig. 5. The time course (h) of the effect of a single s.c. injection of estradiol (25 μg/100 g) on the level of IGF-I transcripts in the uterus of hypophysectomized rats. The level of IGF-I mRNA was determined with a solution RNase protection assay. Values are mean ± SEM. There were five rats, individually analyzed, in each group.

increase at 0.1 μg/100 g (Fig. 4). Progesterone at a dose of 5 μg/100 g had no significant effects.

In the next series of experiments, the time course of estradiol stimulation of IGF-I mRNA accumulation was investigated. Hypophysectomized animals were injected subcutaneously with E_2 (5 μg/100 g) and sacrificed at different times after the injection. The results presented in Fig. 5 show that there was a significant increase of IGF-I mRNA 3 h after estradiol treatment. Furthermore, estradiol increased the levels of IGF-I mRNA in hypophysectomized rats to that of sham-operated rats (Fig. 5).

Discussion

There is now much evidence that endocrine hormones regulate growth factors and growth factor receptors in their target tissues. Several growth factors are present in the uterus and are regulated by steroid hormones (Brigestock et al., 1989). In the present study we characterized the ontogeny and the regulation of IGF-I gene expression during the estrous cycle. The ontogeny of IGF-I differs between tissues and at least three different patterns have been demonstrated (Adamo et al., 1989). The developmental pattern of IGF-I gene expression in the uterus shown in the present study resembles that in the liver, kidney, and muscle with increasing levels postnatally. The expression of IGF-I mRNA in the uterus was clearly estrous cycle-dependent with an 8-fold difference between the lowest levels of expression at diestrus-1 and highest at the day of proestrus. No significant changes of IGF-I mRNA were detected in the corresponding livers demonstrating a tissue specificity in the regulation of IGF-I gene expression. The pattern of IGF-I expression in the uterus differed also from that in the ovary were the lowest levels of expression appeared at the day of proestrus and the highest at estrus and diestrus-1 (Carlsson et al., 1989) suggesting different regulators of IGF-I mRNA expression in these two tissues. Recently, IGF-I receptors were shown to be present in the uterus (Ghahary and Murphy, 1989). Estrogen increases the total number of IGF-I receptors and adult cycling rats displayed a cyclic variation compatible with estrogen dependence in vivo (Ghahary and Murphy, 1989).

According to the original somatomedin hypothesis, IGF-I is a liver-derived mediator of GH action (Daughaday, 1989). More recent studies have demonstrated that IGF-I is produced in many extrahepatic tissues and that several hormones and growth factors can regulate its production (Hsu and Hammond, 1987; Mondschein and Hammond, 1988). Estrogen is more potent than GH in stimulating IGF-I gene expression in the uterus (Murphy et al., 1987). However, there is a possibility that GH and estrogen regulate IGF-I gene expression at different sites in the uterus. Estrogen seems to govern the regulation of IGF-I gene expression during the estrous cycle in the rat, since the cycle variations of IGF-I mRNA follow that of estrogen. GH has been reported to present no estrous cycle-dependent variations in the rat (Clark et al., 1987).

The effect of estradiol is likely to be mediated by estrogen receptors since progesterone was without effect. Furthermore, the dose response of estradiol regulation of IGF-I gene expression presented here is in line with the dose needed to get maximal estradiol binding to nuclear estrogen receptors in the uterus (Anderson et al., 1973). The effect of estradiol was rapid with a significant increase after 3 h. This is in agreement with previous reports on the time course of the regulation of IGF-I (Murphy et al., 1987) and epidermal growth factor-receptor gene expression (Lingham et al., 1988). It is not known whether the effect of estradiol on the abundance of IGF-I transcripts is due to an increase in the rate of transcription of the IGF-I gene or to an increase in stability of the transcript. So far, only GH has been demonstrated to increase the transcription of the IGF-I gene (Mathews et al., 1986).

Estrogen time- and dose-dependently increases IGF-I gene expression and unmanipulated rats displayed an estrous cycle-dependent variation of the levels of IGF-I transcripts that follows the variation of estrogen levels. The estrous cycle-dependent variation in IGF-I gene expression was tissue specific. The regulation of IGF-I gene expression demonstrated here, taken together with the recent demonstration of IGF-I potentiation of estrogen-induced cell proliferation in uterine cells (Ghahary et al., 1990), implies that IGF-I is a modulator of estrogen action in vivo. However,

the more precise physiological role of IGF-I in estradiol action in the uterus remains to be determined.

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Prolactin receptor gene expression in the rabbit: identification, characterization and tissue distribution of several prolactin receptor messenger RNAs encoding a unique precursor

I. Dusanter-Fourt [1], P. Gaye [1], L. Belair [1], B. Pétridou [1], P.A. Kelly [2] and J. Djiane [1]

[1] *Unité d'Endocrinologie Moléculaire, INRA, Centre de Recherches de Jouy-en-Josas, 78350 Jouy-en-Josas, France, and* [2] *Laboratory of Molecular Endocrinology, McGill University, Royal Victoria Hospital, Montreal H3A1A1, Canada*

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Summary

The expression of the prolactin (PRL) receptor gene was studied in rabbit tissues by Northern blot and S1 mapping analysis of mRNA preparations. Rabbit mammary gland contained three major (10.5, 3.4, and 2.7 kb) and one minor (6.2 kb) prolactin receptor poly(A)$^+$ RNA transcripts all of which contain the entire coding sequence of the long form of PRL receptor. Each of these mammary mRNAs hybridized equally well with cDNA sequences encoding either the NH_2 terminal, middle, or COOH terminal part of the rabbit mammary PRL receptor. The four mRNAs differed only in their 5′- and 3′-untranslated regions. The 10.5 kb mammary transcript was further shown to represent a primary transcript of nuclear origin. Among the various rabbit tissues tested, male and female adrenals, mammary gland, ovaries, and jejunum contained the highest level of prolactin receptor mRNA. The prolactin receptor gene was also expressed at moderate to weak abundance in uterus, liver, kidney, pancreas, testis and seminal vesicles. No prolactin receptor mRNA species were detected in adult muscle, lung, total brain, placental cotyledons and spleen, and in thymus from young animals. In all the rabbit tissues examined, the same four PRL receptor poly(A)$^+$ RNA transcripts identified in the mammary gland were expressed and no additional transcript(s) were detected. Variations in the relative proportion of the 10.5 kb transcript and the two smaller transcripts were observed, while the ratio of the 3.4 and 2.7 kb mRNAs remained unchanged. These findings ask for the role of these different transcripts generated in the rabbit, all of which encode the same long form of PRL receptor precursor but have heterogenous 5′- and 3′-untranslated regions. Moreover, they suggest that the various forms of PRL receptor mRNA originate through differential splicing of a single PRL receptor gene.

Introduction

The pituitary polypeptide hormone prolactin (PRL) is involved in a broad spectrum of physio-

Address for correspondence: I. Dusanter-Fourt, Unité d'Endocrinologie Moléculaire, INRA, Centre de Recherches de Jouy-en-Josas, 78350 Jouy-en-Josas, France.

logical processes, producing pronounced biological effects on growth, reproduction and osmoregulation in a wide range of species throughout the vertebrate system (Nicoll and Bern, 1972). In mammals, PRL is primarily responsible for the development of the mammary gland and lactation. PRL initiates its action by interacting with a receptor located in cell membranes (Shiu and Friesen, 1974). Apart from the mammary gland, a variety of mammalian organs contain PRL receptors including adrenal, ovary, testis, liver, and kidney (Posner et al., 1974; Posner and Khan, 1989). After binding of PRL to its receptor, the mechanism(s) by which the hormonal signal is transferred inside the cell remains unknown.

Recently, we first deduced the primary structure of the rat liver prolactin receptor from a single cDNA clone which encodes a mature protein of 291 amino acids (Boutin et al., 1988). We subsequently isolated a PRL receptor cDNA from rabbit mammary gland which encoded a second form of PRL receptor protein, of 592 amino acids. This second PRL receptor type is very similar to the rat liver receptor in its extracellular domain, but with a much longer cytoplasmic domain (Edery et al., 1989). Similarly, a PRL receptor cDNA which encodes a long form of PRL receptor was isolated from human cell lines (Boutin et al., 1989). Rat tissues were recently reported to contain both 'short' and 'long' forms of PRL receptor (Shirota et al., 1990) and a third form of PRL receptor has just been identified in the rat ovary (Zhang et al., 1990). The identification of two different forms of PRL receptor proteins first characterized in the rat liver and the rabbit mammary gland respectively questioned the functions of these two forms and their expression in the rabbit and even within the same tissue. Cloning of the rabbit mammary PRL receptor provides a tool to study the expression of the PRL receptor gene in the rabbit.

In the present paper, we have analyzed the PRL receptor gene expression in the rabbit mammary gland and other tissues and characterized the structural differences of the PRL receptor transcripts. This report indicates that, in the rabbit, four PRL receptor mRNAs are transcribed from a single PRL receptor gene, all of which encode the long form of the PRL receptor only.

Materials and methods

Materials

Enzymes and chemicals were purchased from the following suppliers: DNA polymerase Klenow fragment, T4 polynucleotide kinase, S1 nuclease, and restriction endonucleases from Boehringer Mannheim; deoxyribonucleoside triphosphates (dNTPs) from Pharmacia; avian myeloblastosis virus (AMV) reverse transcriptase from Genofit; [α-^{32}P]dCTP (3000 Ci/mmol), and [α-^{32}P]dATP (3000 Ci/mmol) from Amersham Corp.; guanidium thiocyanate from Fluka; all other chemicals were reagent grade.

Collection of tissues

Tissues from young antigen-immunized male (2-month-old) (thymus), adult male (7-month-old) (testis, seminal vesicles, and adrenals), and pregnant female (27–29-day gestation) (all the other tissues analyzed, including adrenals) New Zealand white rabbits were collected, rapidly cleaned of any adherent material and immediately frozen in liquid nitrogen and stored at $-20\,^{\circ}$C (for classical whole cell extracts' RNA preparation, or microsomes). Pools of two (thymus), four (ovaries, testis, pancreas) and six (adrenals) donors were used for preparation of RNA and microsomes from small organs. For other tissues samples, one donor was used for each preparation of RNA and microsomes and the experiments repeated on 2–6 individual donors. For the preparation of nuclear or polysomal RNA, mammary tissue was freshly used from 29-day gestating animals.

Evaluation of PRL binding sites

Rabbit membranes (microsomes) from the different tissues tested were prepared as previously described (Djiane et al., 1977). 50, 100, and 200 μg of membrane suspension were incubated 16 h at room temperature in the presence of 50,000 cpm of [^{125}I]oPRL, to ascertain linear PRL binding in the different tissues. Specific PRL binding was finally evaluated with 200 μg proteins from every tissue. The specific activity of [^{125}I]oPRL was 50 μCi/μg. Ovine PRL (NIDDK oPRL-16; 30.5 IU/mg) was kindly supplied by the National Hormone and Pituitary Program (Bethesda, MD, U.S.A.).

RNA extraction and Northern blot analysis

RNA was isolated by the guanidium thiocyanate/lithium chloride method (Cathala et al., 1983). Poly(A)$^+$ RNA was isolated by two cycles of oligodeoxythymidylic acid cellulose chromatography (Aviv and Leder, 1972). 20 μg of poly(A)$^+$ RNA was denatured in 50% (v/v) formamide and 2.2 M formaldehyde, size separated by electrophoresis on 1% (w/v) agarose gels containing 2.2 M formaldehyde and then blotted onto nitrocellulose-nylon matrix (Hybond C extra, Amersham) (Thomas, 1980). Filters were baked for 2 h at 80 °C and then prehybridized in solution made of 50% (v/v) deionized formamide, 5 × Denhardt's solution (1 × Denhardt = 0.02% w/v each of bovine serum albumin, Ficoll, and polyvinylpyrrolidone), 5 × standard saline phosphate EDTA (SSPE) (1 × SSPE = 1.15 M NaCl-0.01 M NaH_2PO_4-1 mM EDTA), 250 μg/ml denaturated salmon sperm DNA, and 0.1% sodium dodecyl sulfate (SDS), for at least 3 h. The filters were then hybridized with the indicated cDNA probes generated by suitable endonuclease digestion from the PRL receptor cDNA PRL R2-4 or PRL R2-2 previously isolated (Edery et al., 1989), labeled with ^{32}P by random priming to a specific activity of 10^8 cpm/μg and used at approximately 0.5–1 × 10^6 cpm/ml. Hybridizations were performed at 42 °C for 24 h in the presence of prehybridization solution containing 6% dextran sulfate. At the end of the hybridization period the blots were washed once in 0.1 × SSC (1 × SSC = 0.15 M NaCl-0.015 M sodium citrate), 0.1% SDS, and 1 mM EDTA for 10 min at room temperature, followed by two washes in 0.1 SSC-0.1% SDS-1 mM EDTA for 30 min at 60 °C. Filters were exposed to Kodak X-Omat S film at −70 °C with intensifying screen. When indicated, hybridization signals were quantified by densitometric scanning of multiple autoradiograms of various exposures of the similar RNA samples.

Nuclear RNA was prepared according to Brown and Rosen (1986), and polysomal RNA was extracted according to Shapiro and Young (1981) without further isolation of poly(A)$^+$ RNA. 20 μg were loaded on the gel, and a Northern blot analysis was carried out as above. RNA molecular sizes were estimated with an RNA ladder (BRL, range 0.24–9.5 kilobases) simultaneously loaded on the gel and stained with ethidium bromide before blotting.

Genomic DNA was prepared from rabbit liver and Southern blot analysis was performed using conventional techniques (Sambrook et al., 1989).

S1 nuclease analysis

Suitable restriction fragments of the PRL receptor cDNA PRL R24 were subcloned into M13 mp18 and M13 mp19 derivatives, as described previously (Edery et al., 1989). A non-universal M13 primer MIM (5′-CGCCAGGGTTTTCCC-3′) was synthesized in an Applied Biosystem synthesizer, and was annealed to single-stranded M13 subclones. Klenow fragment of *Escherichia coli* DNA polymerase was added (1 U/μg DNA) to the annealed mixture in the presence of 50 μCi [α-^{32}P]dCTP (3000 Ci/mmol, Amersham) and 200 μM each of dATP, dGTP, dTTP, in order to obtain a uniformly labeled probe, as described (Sambrook et al., 1989). Double-stranded DNA was further digested with the indicated restriction endonuclease. The labeled probe was separated from the M13 template by electrophoresis in 4–6% polyacrylamide, 8 M urea gels and recovered from the gel by mechanical elution. An aliquot of the labeled cDNA probe (150,000–200,000 cpm) and RNA were coprecipitated, hybridized overnight in 80% deionized formamide, 40 mM Pipes pH 6.4, 400 mM NaCl, 1 mM EDTA, and further digested with S1 nuclease (150 U) in 200 μl digestion mixture containing 200 mM NaCl, 30 mM Na acetate pH 4.4, 4.5 mM $Zn(CH_3COO)_2$, as described (Sambrook et al., 1989). The reaction was stopped with 50 mM ammonium acetate, 5 mM EDTA and the final products were subjected to 4 or 6% polyacrylamide, 8 M urea gel electrophoresis, depending on the size of the initial cDNA probe. *Hae*III fragments from PhiX174 RF (BRL) were used as size markers after 5′-end labeling using conventional procedures (Sambrook et al., 1989).

Primer extension experiment

Primer extension experiments were performed essentially as described by Bennett et al. (1989). The 22-mer oligonucleotide primer (5′-GGAA-GACAATCATGGATGCCAC-3′) was synthesized using a 8600 Milligen Biosearch apparatus,

and further purified by polyacrylamide gel electrophoresis. Primer was labeled at its 5′-end by using [α-^{32}P]dATP and T4 polynucleotide kinase as described (Sambrook et al., 1989).

Results

Multiple forms of mammary prolactin receptor mRNAs

PRL receptor gene expression was first studied in rabbit mammary gland by Northern blot analysis, using as a probe the PRL receptor cDNA clone PRL R2-2 (~ 1600 base pairs (bp)) recently characterized (Edery et al., 1989). This probe encodes the NH_2 terminal extracellular part of the mammary PRL receptor (~ 340 amino acids), and spans the entire 517 nucleotide 5′-untranslated region previously described (see the top drawing in Fig. 1). Three major transcripts of 10.5, 3.4, and 2.7 kilobases (kb), and one minor transcript of 6.2 kb (Fig. 1*A*) were identified in poly(A)$^+$ RNA preparations from mammary gland. Using densitometric scanning analysis, the relative proportion of each PRL receptor transcript was evaluated. When expressed as a fraction of whole PRL receptor mRNAs, the 10.5, 6.2, 3.4, and 2.7 kb mRNA species represented 24 ± 9%, 5 ± 3%, 30 ± 5%, and 40 ± 7% respectively (mean ± SEM of six independent experiments). Great variability of the signal intensity for the 10.5 kb mRNA species was observed. Analysis of nuclear and cytoplasmic polysomal RNA from mammary tissue was carried out to identify the subcellular origin of the different PRL receptor transcripts. PRL receptor mRNA sizes identical to the species seen in the whole cell extract (10.5, 6.2, 3.4, and 2.7 kb) were identified in both nuclear and polysomal RNA preparations (not shown). However, relative intensities of the receptor transcripts differed notably: as shown in Fig. 1*C*, the 10.5 kb transcript was greatly enhanced in the nuclear RNA fraction and barely detectable in the polysomal RNA preparation, suggesting it represents a primary nuclear RNA transcript.

All PRL receptor mRNA transcripts identified above are of sufficient length to encode the 'long' form of the PRL receptor precursor, since the rabbit mammary PRL receptor cDNA contained a 1848 nucleotide open reading frame (Edery et al., 1989). However, several partial cDNAs specific to various regions of the rabbit mammary PRL receptor cDNA were used to probe for the presence of one or more mammary transcripts encoding a short form of the PRL receptor. As shown in Fig. 1*A*, lanes b–d, all the four PRL receptor mRNAs hybridized to all the different probes throughout the entire coding region and in the 5′-untranslated region (UTR) of the mammary cDNA. In contrast, the two smaller transcripts (2.7 and 3.4 kb) did not hybridize to probes 1e and 1f from the distal 3′ UTR of PRL receptor cDNA. To firmly establish that no rabbit mammary PRL receptor transcripts encode a PRL receptor molecule of the short type, S1 nuclease protection experiments were also carried out. Different single-stranded probes within the rabbit mammary PRL receptor coding sequence were synthesized using the M13 vector. As shown in Fig. 2, the P lanes identify the original size of the probe used, and open arrowheads indicate the totally protected cDNA fragments expected from each probe, i.e. deleted from the additional M13 vector polylinker region (45–70 nucleotides). None of these probes was protected when lung RNAs were used in the S1 nuclease analysis. In contrast, the cDNA fragments from the probes synthesized were all entirely protected in the presence of either total (not shown) or poly(A)$^+$ mammary RNA. Fainter protected fragments were identified occasionally (see Fig. 2, lane d) and were shown to reflect radiolysis and prove degradation only. These results clearly indicate the absence of any mammary transcript encoding a different PRL receptor type than the long form originally described (Edery et al., 1989).

3′ UTR sequence analysis indicated the presence of at least one polyadenylation signal (AATAAA) at position +2119 bp followed by a consensus sequence (CATTG) frequently observed downstream of polyadenylation recognition sites in vertebrate genes (Benoist et al., 1980). The use of this potential site for 3′-end processing predicts a PRL receptor mRNA of 2400 to 2600 bases depending on the 5′ UTR size (see above) which would correspond well with the smaller 2.7 kb PRL receptor mRNA identified.

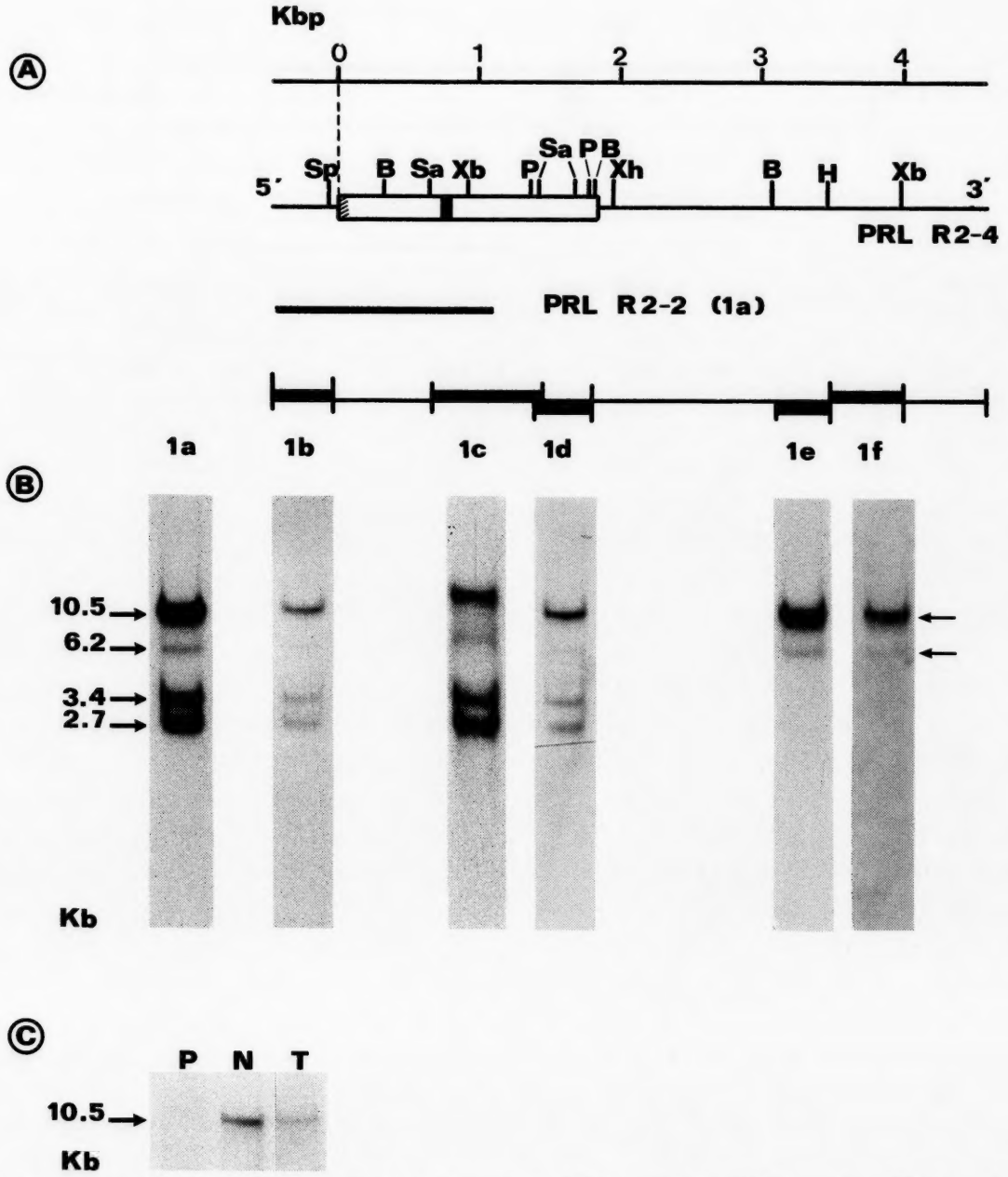

Fig. 1. Northern blot analysis of rabbit mammary RNA with various PRL receptor cDNA probes. The structure of the mammary PRL receptor cDNA is depicted in panel *A*. The open reading frame encoding the long form of the PRL receptor protein is boxed. The transmembrane domain (solid) as well as the peptide signal region (stripped) are shown. The various PRL receptor cDNA fragments used as probes in experiments shown in *B* and *C* are outlined below. *B*: 20 μg of poly(A)$^+$ RNA were applied to a 1% agarose-2.2 M formaldehyde gel. This blot was successively hybridized to the appropriate labeled probe 1a spanning the first ~1600 bp of the 5′-terminal region (−517 to ~ +1020), probe 1b spanning the first 452 bp of the 5′-terminal region to the *Sph*I site (−517 to −65), probe 1c spanning the *Sac*I-*Sac*I region (+666 to +1425), probe 1e spanning the *Bam*HI-*Hin*dIII region (~ +3070 to ~ +3500), and probe 1f spanning the *Hin*dIII-*Xba*I region (~ +3500 to ~ +4050) of the PRL receptor cDNA, nucleotides being numbered from the first base of the initiator codon. Probe 1d spanning the *Pst*I-*Pst*I region (+1385 to +1788) was hybridized to another blot where poly(A)$^+$ RNA had migrated more slowly; RNA molecular sizes were estimated with an RNA ladder. *C*: RNA was isolated from polysomal (P), nuclear (N), or total (T) cell fractions, and 20 μg were applied to a 1% agarose-formaldehyde gel as above. Northern blot analysis was performed using probe 1a. Only the 10.5 kb band is shown. Abbreviations used for restriction endonucleases: Sp = *Sph*I; B = *Bam*HI; Sa = *Sac*I; Xb = *Xba*I; P = *Pst*I; Xh = *Xho*I.

Additional heterogeneity at the 5′-end of the rabbit mammary PRL receptor mRNA transcripts

To obtain additional information on the structural relationships between the four PRL receptor mRNA transcripts, S1 mapping and primer extension experiments were carried out in the 5′-untranslated region. A single-stranded cDNA probe spanning 452 nucleotides of the 517 nucleotide 5′

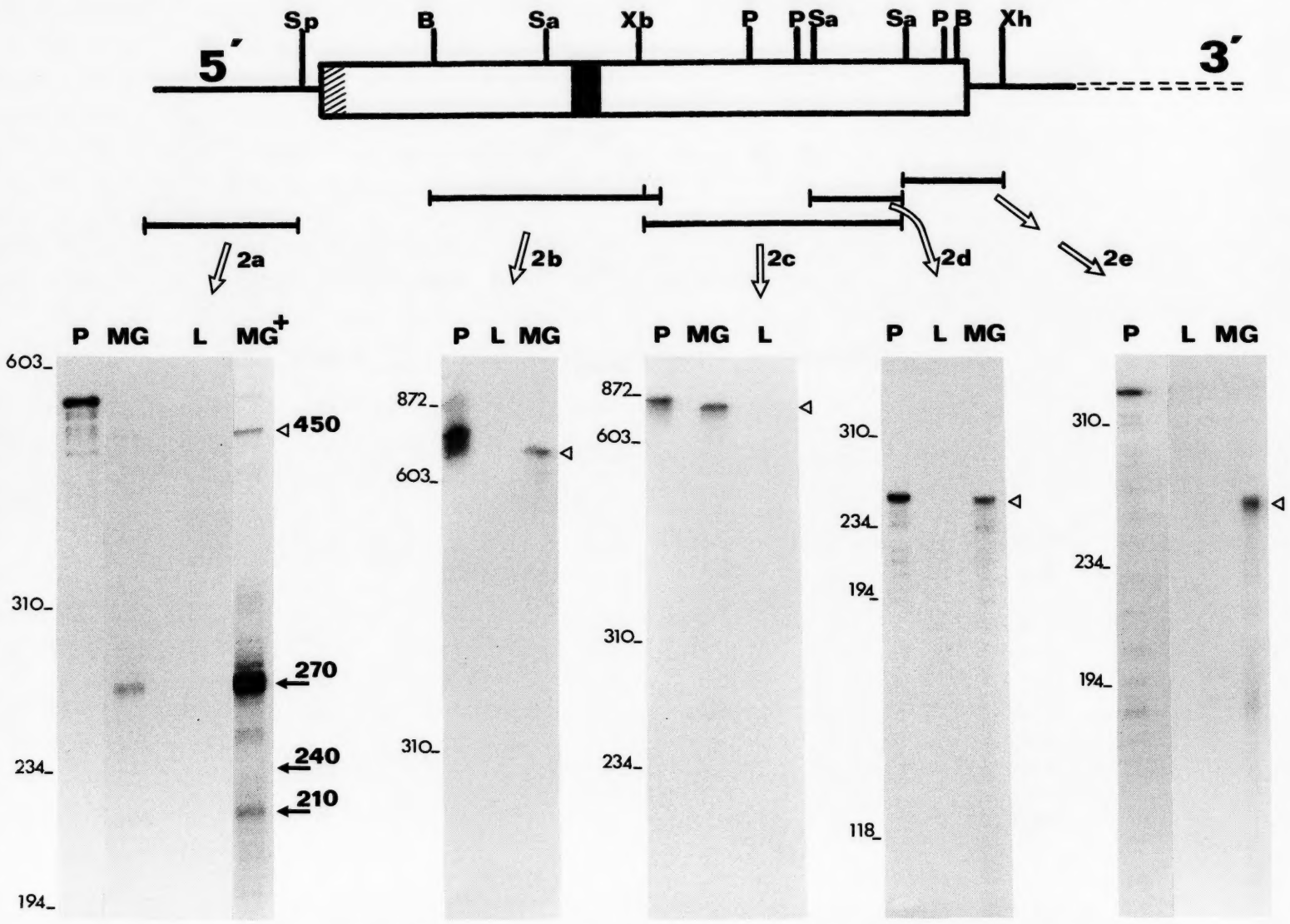

Fig. 2. S1 nuclease analysis of rabbit mammary RNA with various PRL receptor cDNA probes. Various PRL receptor cDNA fragments (2a–2e) were inserted into the M13 vector in the appropriate orientation detecting sense (+). Single-stranded probes were synthesized, namely probe 2a spanning the first 5′-terminal 452 nucleotides (−517 to −65), probe 2b spanning the *Bam*HI-*Xba*I region (670 nucleotides, from +341 to +941), probe 2c spanning the *Xba*I-*Sac*I region (738 nucleotides, from +941 to +1679), probe 2d spanning the *Sac*I-*Sac*I region (254 nucleotides, from +1425 to +1679), probe 2e spanning the *Sac*I-*Xho*I region (260 nucleotides, from +1679 to +1939) of the PRL receptor cDNA, as indicated in the drawing. Each of these probes (shown in lanes P respectively) was annealed to mammary gland (MG) or lung (L) poly(A)$^+$ RNA (20 μg) and subjected to S1 nuclease digestion. Products were analyzed by 4 or 6% polyacrylamide gel electrophoresis depending on the probe size. The completely protected PRL receptor cDNA fragments expected from each probe are indicated by open arrows in each panel. Size markers (^{32}P-labeled *Hae*III fragments from PhiX174) are shown in the left margin of each autoradiogram.

UTR of the PRL receptor cDNA (see drawing in Fig. 2) was synthesized using the M13 vector as above. As shown in Fig. 1*B*, lane b, this probe recognized all four PRL receptor transcripts. From this 5′ UTR probe, only 270 nucleotides were protected and commonly expressed by the majority of the mammary PRL receptor transcripts (Fig. 2a, lane MG). In addition, various minor fragments of 450, 240, and 210 nucleotides, respectively were protected by mammary mRNAs and detected after prolonged exposure (Fig. 2*A*, lane MG$^+$). Autoradiographic intensities of the protected fragments reflect the relative amount of each protecting mRNA as a part of the whole PRL receptor transcripts. As analyzed by densitometric scanning, > 80% of the PRL receptor transcripts protected 270 nucleotides of the probe, while only 5% of the receptor transcripts protected

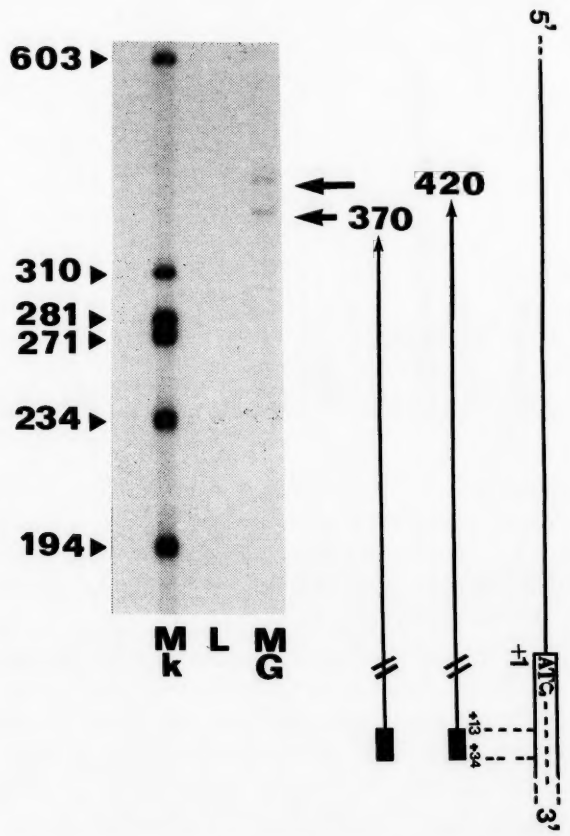

Fig. 3. Primer extension analysis of the 5′-untranslated region of mammary PRL receptor mRNA. 20 μg of mammary (MG) or lung (L) poly(A)$^+$ RNA were used to extend the 5′-end-labeled 22-mer oligonucleotide primer complementary to the +13 to +34 nucleotide region of the PRL receptor cDNA, as indicated in the right drawing. Extension products were analyzed in 6% polyacrylamide gel. Unextended primer was not visible in the portion of the autoradiogram shown here. Size markers (lane Mk) (^{32}P-labeled *Hae*III fragments of PhiX174) are indicated in nucleotides in the margin of the figure.

the entire 450 nucleotide fragment corresponding to the 5′ UTR of the PRL receptor cDNA that was isolated.

We further performed primer extension experiments to fully characterize the heterogeneity and structural divergence in the 5′-untranslated region of the mammary PRL receptor transcripts. In the presence of mammary poly(A)$^+$ RNA, two extended products, of ~ 370 and ~ 420 nucleotides respectively, were obtained in roughly equal quantities by primer extension of a 22-mer oligonucleotide complementary to the beginning of the coding region of PRL receptor cDNA (nucleotide +13 to nucleotide +34 from the initiator codon, Fig. 3). These two cDNA species are the result of specific priming, since no extended products were obtained in the presence of lung RNA (lane L). Taking into account the presence of 34 nucleotides from the coding sequence in the two extended cDNA products, these results indicate that two different 5′-untranslated regions, of 330 nucleotides and at least 390 nucleotides, respectively, were expressed in the mammary PRL receptor transcripts. Correspondence of the band sizes identified by this primer extension experiment and the S1 nuclease protection assay of the 5′ UTR region depicted in Fig. 2a were analyzed. Since 97 nucleotides separated the 3′-end of the S1 probe 2a (*Sph*I site) and the 3′-end of the oligonucleotide primer, the extended 370 nucleotide cDNA product (Fig. 3) exactly reflected the major 270 nucleotide S1 protected fragment seen in Fig. 2a. The extended 420 nucleotide cDNA product identified would reflect a 320 nucleotide S1 protected fragment.

These results clearly establish a high degree of heterogeneity at the 5′-end of the rabbit mammary PRL receptor mRNAs, in addition to the divergence in the 3′ UTR observed in Fig. 1, probe 1e–1f. Whether all the mammary PRL receptor transcripts identified were transcribed from one or more PRL receptor gene(s) was next investigated by Southern blot analysis on rabbit genomic DNA.

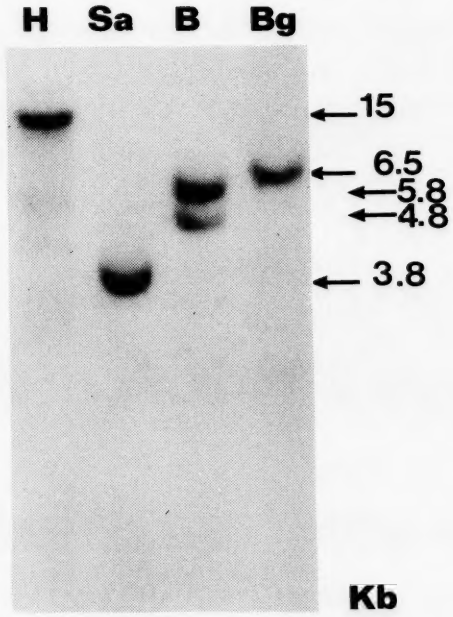

Fig. 4. Southern blot analysis of endonuclease-digested rabbit genomic DNA. 20 μg of genomic DNA were digested with the indicated restriction endonucleases, loaded on a 1% agarose gel, and transferred on nylon membrane. Blot was hybridized to the probe 1c (Fig. 1) and exposed for 4 days. Abbreviations: H = *Hin*dIII; Sa = *Sac*I; B = *Bam*HI; Bg = *Bgl*II.

As shown in Fig. 4, the 0.8 kb cDNA probe 1c (in the coding sequence, see Fig. 1) detected single genomic DNA fragments from either *Hin*dIII (H), *Sac*I (Sa) or *Bgl*II (Bg)-digested genomic DNA. Similarly, the 0.5 kb cDNA probe 1e (5′ UTR) detected a single 5 kb genomic DNA fragment from *Hin*dIII-digested genomic DNA (not shown). These results suggest the presence of a single PRL receptor gene in the rabbit genome.

Distribution of PRL receptor mRNA in rabbit tissues

PRL receptor mRNA abundance and size were assessed in various rabbit tissues by S1 nuclease protection and Northern blot analysis respectively. S1 mapping analysis was performed with the 670 nucleotide single-stranded DNA probe 2b encoding the well-conserved extracellular and transmembrane domains of the PRL receptor molecule (see Fig. 2). Using such a technique, the overall expression of the PRL receptor gene could be determined independently of the actual type(s) or size(s) of the different PRL receptor RNAs produced. As illustrated in Fig. 5, the various RNA preparations studied protected the cDNA probe to variable extent. However, RNAs from all these tissues completely protected the cDNA probe. No partially protected DNA fragments were identified, indicating the absence of heterogeneity in the 5′-coding region of the rabbit PRL receptor gene expressed. Using densitometric scanning, the whole PRL receptor RNA content was thus evaluated in each organ and compared to

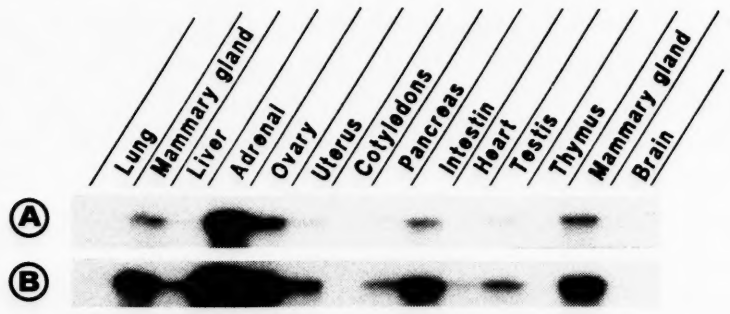

Fig. 5. S1 nuclease analysis of PRL receptor RNA abundance in various rabbit tissues. 80 μg of total RNA extracted from each rabbit tissue indicated were annealed to the single-stranded 670 nucleotide DNA probe 2b depicted in Fig. 2 and subjected to S1 nuclease digestion. *A* shows a 5 h exposure (−70 °C) and *B* shows an 18 h exposure (−70 °C) of the same analytical gel. Adrenals were from male animals. A single representative experiment is shown.

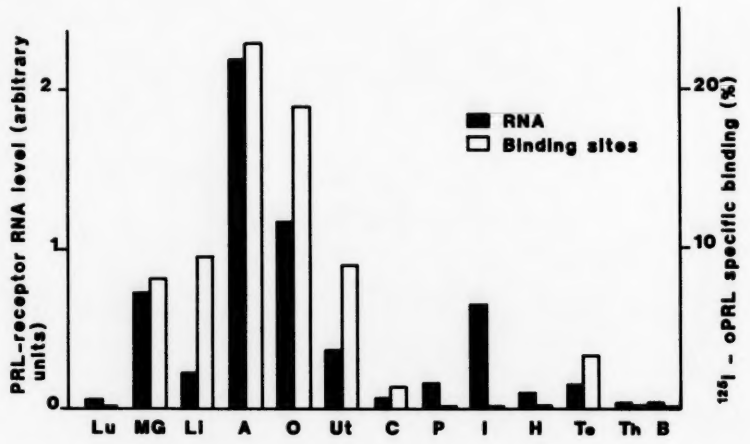

Fig. 6. Expression of PRL receptor in rabbit tissues: comparison of steady-state PRL receptor RNA and PRL binding levels. Total PRL receptor RNA level expressed in each tissue studied was evaluated relative to the intensity of the PRL receptor RNA-cDNA duplex protected in the S1 nuclease experiment depicted in Fig. 4, as quantified by densitometric scanning. PRL binding levels were expressed as a ratio of the specific [^{125}I]oPRL binding obtained in each tissue and the total [^{125}I]oPRL added in the incubation medium. PRL binding and PRL receptor RNA were estimated on tissue samples from the same donors. Abbreviations: mammary gland (MG), liver (Li), adrenal (A), ovary (O), kidney (K), heart (H), muscle (M), pancreas (P), placental cotyledons (C), intestinal jejunum (I), lung (Lu), thymus (Th) and testis (Te). One representative experiment is shown.

PRL binding data (Fig. 6). Highest levels of PRL receptor RNAs were identified in four tissues, namely male and female adrenals, mammary gland, ovaries, and intestine (jejunum). Moderate to weak levels were observed in uterus, liver, pancreas, kidney, testis, and seminal vesicles (not shown). No, or extremely low levels of PRL receptor RNAs (see the overexposed autoradiogram in Fig. 5*B*) were detected in heart, lung, muscle and spleen (not shown), total brain, placental cotyledons, and thymus from young immunostimulated animals. These results were in good agreement with PRL binding data (open bars), except for the intestine where high level of PRL receptor RNA was strikingly opposed to undetectable level of PRL binding sites in the same tissue sample. However, moderate divergence between RNA and binding levels did occur: liver, ovary, and uterus showed PRL receptor RNA levels substantially higher than expected from binding data while PRL receptor RNAs were unexpectedly detectable in the pancreas where no PRL binding sites were measurable.

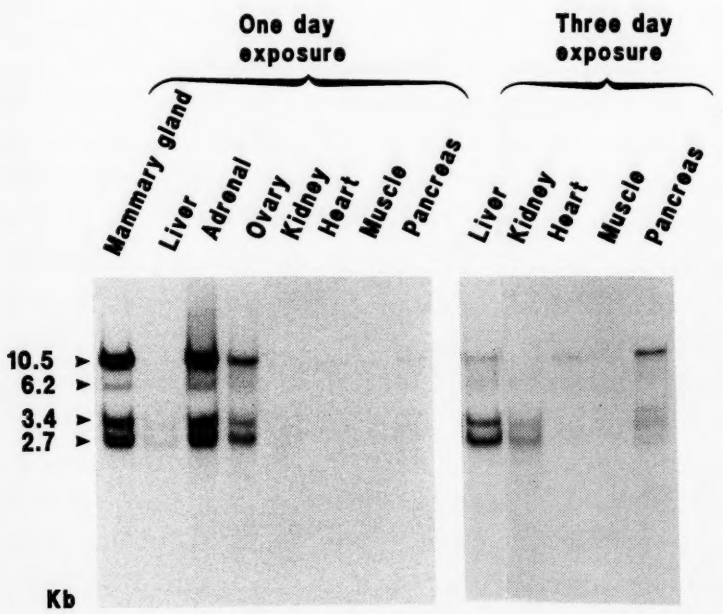

Fig. 7. Northern blot analysis of PRL receptor RNA from various rabbit tissues. Northern blot analysis was performed as described in the legend to Fig. 1, using the probe 1a and 20 μg of poly(A)$^+$ RNA from each tissue sample. A 1-day and a 3-day exposure ($-70\,^\circ$C) of the same poly(A)$^+$ RNA Northern blot are shown. Adrenals were from 27-day-pregnant rabbits. RNA molecular sizes were estimated with an RNA ladder.

Northern blot analyses were performed in order to characterize the PRL receptor RNA transcripts in the various rabbit tissues. As shown in Fig. 7, all the rabbit tissues expressing PRL receptor mRNAs exhibited the same three predominant PRL receptor poly(A)$^+$ RNA transcripts (10.5, 3.4, and 2.7 kb) identified in the mammary gland (see above). Minor transcripts such as the 6.2 kb species were also detected. Similar results were obtained in the intestine (jejunum), testis and uterus (not shown). Interestingly, no additional poly(A)$^+$ RNA transcripts were detected in any of the tissues analyzed, even after prolonged exposure. While the relative amount of the two lower (2.7 and 3.4 kb) RNA transcripts was strikingly constant in the different tissues, the proportion of the 10.5 kb PRL receptor RNA transcript did vary specifically between tissues, representing almost the entire PRL receptor gene expression in some cases (see the pancreas, Fig. 7).

Discussion

Transcription of the PRL receptor gene and processing of the receptor mRNA appear as a complex phenomenon that generated multiple mRNA transcripts in rabbit tissues. Four PRL receptor poly(A)$^+$ RNA species were first detected in the mammary gland, of 2.7, 3.4, 6.2, and 10.5 kb. While these rabbit mammary transcripts were similar in size to those species identified in human tissues (2.8, 3.5, and 7.3 kb) (Boutin et al., 1989), they were strikingly different from the unique smaller transcript of about 2 kb originally identified in rat liver and expressed in many rat tissues (Boutin et al., 1988), or mice liver (2.5 kb) (David and Linzer, 1989), suggesting the existence of at least two groups of species expressing the PRL receptor gene differently. As demonstrated by Northern blot analysis and S1 nuclease mapping with various cDNA probes, each of the four mammary PRL receptor mRNA species contained the entire sequence coding for the 'long' form of the PRL receptor identified in the rabbit. Therefore, under our experimental conditions, no expression of a 'short' PRL receptor such as that identified in the rat liver, could be detected. This result is entirely different from recent data demonstrating that the rat tissues can contain both 'short' and 'long' forms of PRL receptor (Shirota et al., 1990). Interestingly, in this last species, the proportion of short/long form varies from tissue to tissue (Shirota et al., 1990; G. Jahn, submitted). In the rat ovary where the long form in this species was first detected (Shirota et al., 1990), there may in fact be a third form of the PRL receptor (Zhang et al., 1990). Another important species difference, based on Northern blot analysis of poly(A)$^+$ RNA from various rabbit tissues, is the strikingly uniform expression of the PRL receptor gene in the rabbit compared to data reported from rat tissues where different transcripts were specifically expressed in different tissues (Boutin et al., 1988). Taken together, these results demonstrate that the expression of the PRL receptor gene is very different in the rabbit and the rat, further strengthening the concept of a strong species-specific regulation of PRL receptor gene expression.

The exact nature of these different PRL receptor mRNA species was further investigated and shown to reside in both the 5′- and 3′-untranslated regions. First of all, primer extension analysis indicated the existence of two different and

equally expressed 5′ UTR regions among mammary PRL receptor mRNA, of 330 and at least 390 nucleotides. However, we could not ascertain whether the primer extension along the mammary PRL receptor transcripts was complete: prolonged extension of the primer might have been hampered, by secondary structures for instance, and the size of the longer extended product might be underestimated. S1 mapping experiments on the total 5′ UTR region indicated the existence of additional important 5′-heterogeneity among the transcripts, since only 5% of the PRL receptor transcripts contain the 5′ UTR of the PRL receptor cDNA originally isolated and > 80% protected the short 5′ UTR identified by the primer extension analysis. Taken together, these results suggest that half of the PRL receptor mRNAs are characterized by a short 330 nucleotide-long 5′-untranslated region while 30% of the PRL receptor transcripts exhibit a longer 5′-untranslated region which contains the short 330 nucleotide-long 5′ UTR and an additional upstream nucleotide sequence different from the 5′ UTR of the PRL receptor cDNA isolated. Even if the primer extension along the mammary mRNA was total, primer extension of the transcripts that contain the 500 nucleotide-long 5′ UTR of the PRL receptor cDNA isolated might have been undetected because of the very small percentage (5%) of these transcripts. Since Southern blot analysis on rabbit genomic DNA (Fig. 4) and cloning of the rat PRL receptor gene (D. Banville, personal communication) indicate the existence of a single PRL receptor gene in these two species, the different rabbit PRL receptor mRNAs identified might be generated through differential splicing of the PRL receptor gene. Whether, in addition to alternative usage of exons, there may be an alternative usage of promoters is presently unknown. Such mechanisms are very often tissue-specific and provide the cell with an additional control mechanism for gene expression to changes in environment and generate diversity from single genes (Darnell, 1982; Andreadis et al., 1987). Heterogeneity of the 5′-untranslated region was also described for the growth hormone receptor (Leung et al., 1987; Mathews et al., 1989) and recently observed for the rat PRL receptor (Shirota et al., 1990). This heterogeneity of the 5′ UTR could not solely account for the great variation in mammary PRL receptor mRNA sizes we observed (from 2.7 to 6.2 kb at least). As indicated in Fig. 1, the two smaller receptor transcripts (2.7 and 3.4 kb) did not hybridize to distal 3′ UTR cDNA probes, and analysis of the 3′ UTR in the cDNA sequence used indicates the presence of at least one polyA addition signal, suggesting the use of alternate 3′-polyA addition-cleavage sites and/or 3′-end processing accounting for these mRNA size variations. Whether differential splicing in that 3′-non-coding region also occurs remains to be established. Further analysis of these 5′ and 3′ UTR awaits the availability of appropriate genomic clones. Independently of the actual mechanism giving rise to the 5′- and 3′-end heterogeneity of the PRL receptor mRNA, the fact that this heterogeneity is confined to the untranslated region of the mRNAs opens the possibility that these different forms of mammary PRL receptor mRNA are subjected to translational control (Pelletier and Sonenberg, 1985; Theil, 1990). There are numerous examples of regulation of gene expression through reversible translational regulation and in many of these cases, translational control of gene expression has been shown to depend on specific features of the primary and/or secondary structure of mRNA, such as stem loop structures and small open reading frames upstream of the main coding sequence (Kozak, 1986, 1987, 1989; Miller and Hinnebush, 1989). It is worth noting that such upstream sequences do exist in all PRL receptor cDNAs isolated from various species (Boutin et al., 1988; David and Linzer, 1989; Zhang et al., 1990) and in numerous other homologous hormone receptor cDNAs isolated (Kozak, 1987; Leung et al., 1987; Gearing et al., 1989; Mathews et al., 1989).

PRL receptor gene expression was studied in various rabbit tissues, using S1 nuclease protection. While the highest PRL receptor gene expression identified for the mammary gland, adrenal and ovary, was predicted by binding data (Posner et al., 1983) (Fig. 6) the large amount of PRL receptor mRNA in intestine was strikingly opposed to PRL binding data in this tissue. So far, the presence of PRL receptors in the intestine has only been reported for membrane fractions from the gut of several amphibians (White, 1981) while we failed to observe PRL binding sites in the

rabbit jejunum microsomes. However, many previous reports had pointed out the importance of PRL actions on electrolyte and nutrient transport across the small intestine of the rat, guinea pig and hamster (Ramsey and Bern, 1972; Mainoya, 1975a, b). Recently, absorption and transepithelial transport of PRL was identified in jejunum and ileum in suckling rats (Gonella et al., 1989). Therefore the present identification of a high level of PRL receptor gene expression in the jejunum gives further support to a specific role of PRL in gut functions. The absence of detectable PRL binding sites in the same intestinal tissue sample where PRL receptor gene was highly expressed suggests either the existence of a potent inhibition of translational control (see above) of PRL receptor expression in that tissue, in response to still unknown transient biological signal(s) or the existence of a truely expressed intestinal PRL receptor protein that is rapidly degraded and/or undetectable by conventional PRL binding assays. Preliminary data favor this last hypothesis since we have recently detected PRL binding sites in freshly isolated epithelial cells (unpublished).

The moderate to weak expression of PRL receptor gene detected in uterus, liver, pancreas, testis, kidney and seminal vesicles (these last two tissues not being shown), and heart (barely detectable) was in good agreement with PRL binding data (Fig. 5) (Posner et al., 1974; Posner and Khan, 1983). Moderate divergence between binding and RNA levels did occur, and these discrepancies might be explained as mentioned above. The detectable expression of PRL receptor gene in pancreatic tissue is worth noting, taking into account the recent observations of important PRL effects on the development and insulin secretion from islets of Langerhans (Bredje et al., 1989). In that tissue the major PRL receptor transcript was the previously identified nuclear 10.5 kb species. Whether such a transcript represents a biologically regulated step of PRL receptor RNA maturation, this transcript being maintained non-functional by temporarily retaining at least part of one intron as described in some cases (Kozak, 1988), remains to be established.

No expression of the PRL receptor gene was detected in muscle, lung, placental cotyledons, spleen, total brain and thymus. These last two results deserve comments. Although PRL binding sites were previously identified in brain (Di Carlo and Muccioli, 1981; Posner and Khan, 1983), they were only detected in specific areas of the rabbit brain suggesting the absence of detectable PRL binding sites, and PRL receptor RNA in the overall organ (Di Carlo et al., 1984). The absence of PRL receptor expression in the thymus of young immunostimulated rabbits was more surprising since numerous reports had suggested an immunoregulatory role for PRL, involving modulation of T lymphocytes (Russell et al., 1985; Hierstand et al., 1986; Bernton et al., 1988) and direct control of thymic hormonal production (Dardenne et al., 1989). Preliminarily observations have localized PRL receptors in a thymic epithelial cell subpopulation which only represents a minority of the whole thymic cell population (M. Dardenne and P.A. Kelly, unpublished results).

In conclusion, the present results indicate that four PRL receptor mRNAs are transcribed from a single PRL receptor gene in the rabbit, all of which encode a unique long form of the PRL receptor. The physiological role(s) of these multiple rabbit PRL receptor transcripts which only differ in their 5′ and 3′ UTR remains to be established.

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Medium hyperosmolarity depresses thyrotropin-releasing hormone-induced Ca^{2+} influx and prolactin secretion in GH_4C_1 cells

Noriyuki Sato, Xiangbing Wang and Monte A. Greer

Section of Endocrinology, Department of Medicine, Oregon Health Sciences University, Portland, OR 97201, U.S.A.

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Key words: Hyperosmolarity; Secretion; Cytosolic Ca^{2+} concentration; (GH_4C_1 cells)

Summary

We studied the influence of graded degrees of hyperosmolarity on the dynamics of the thyrotropin-releasing hormone (TRH)-induced rise in cytosol Ca^{2+} concentration ($[Ca^{2+}]_i$) and prolactin (PRL) secretion in GH_4C_1 cells. TRH caused two phases of increase in $[Ca^{2+}]_i$ that were differentially altered by hyperosmolarity: 100% hyperosmolarity (600 mOsm) depressed only 20% of an initial high-amplitude $[Ca^{2+}]_i$ burst (first phase) dependent on Ca^{2+} mobilized from intracellular pools, but it abolished a sustained low-amplitude second phase dependent on extracellular Ca^{2+} influx. Low degrees of hyperosmolarity suppressed PRL secretion due to Ca^{2+} influx while high degrees suppressed secretion due to mobilized Ca^{2+}. These data suggest that in GH_4C_1 cells hypertonic inhibition of secretion may result from both blocking Ca^{2+} influx and mechanisms unrelated to $[Ca^{2+}]_i$.

Introduction

It has long been recognized that maintaining extracellular osmolarity isotonic is an important factor for stable hormone secretion both in vitro and in vivo. In this decade it is becoming clear that osmolarity exerts an important direct influence on exocytosis (Rink and Knight, 1988). Medium hyposmolarity stimulates exocytosis (not only hormone secretion from rat pancreatic islet (Blackard et al., 1975; Murakami et al., 1986), adenohypophyseal (Greer et al., 1983, 1990), and pituitary tumor-derived GH_4C_1 cells (Sato et al., 1990d, e) but also lysosomal enzyme release from human polymorphonuclear leukocytes (Sato et al., 1990a)). In this phenomenon, the induced cell swelling, not extracellular hyposmolarity, appears the primary factor in stimulating secretion since other methods of inducing cell swelling such as changing medium osmolarity from hypertonic to isotonic (Greer et al., 1985) or adding isotonic permeant molecules (urea, glycerol, and ethanol (Wang et al., 1989; Sato et al., 1990b, c)) produce the same effect. Ca^{2+} plays an important role in this phenomenon; removal of medium Ca^{2+} enhances cell swelling-induced hormone secretion in normal rat adenohypophyseal cells (Greer et al., 1990) but abolishes induced prolactin (PRL) secretion in GH_4C_1 cells (Sato et al., 1990b, c, d).

Conversely, medium hyperosmolarity inhibits secretion by adenohypophyseal (Greer et al., 1985),

Address for correspondence: Monte A. Greer, M.D., Section of Endocrinology, Department of Medicine, Oregon Health Sciences University, Portland, OR 97201, U.S.A.

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parathyroid (Brown et al., 1978), pancreatic islet (Murakami et al., 1986), and adrenal chromaffin cells (Hampton and Holz, 1983; Pollard et al., 1984). This depressive effect of hyperosmolarity on hormone secretion has been interpreted as a direct effect on membrane fusion during exocytosis (Holz, 1986). However, the effect of hyperosmolarity on second messenger systems has not been delineated. We have therefore studied the influence of medium hyperosmolarity on the rise in cytosolic Ca^{2+} concentration ($[Ca^{2+}]_i$) and PRL secretion induced by thyrotropin-releasing hormone (TRH) in GH_4C_1 cells. GH_4C_1 cells were chosen for the investigation because we wished to have a uniform cell population to study and Ca^{2+} plays a very important role in secretagogue-stimulated hormone secretion (Gershengorn, 1986) and cell volume changes (Sato et al., 1990b) in these cells.

Materials and methods

Medium (pH 7.4, 300 mOsm) was Krebs-Ringer Hepes buffer (KRBH); the composition (mM) is NaCl (140), KCl (5), $MgSO_4$ (1.2), KH_2PO_4 (1.2), glucose (6), Hepes (10), $CaCl_2$ (1.5), and 0.1% bovine serum albumin (BSA). Ca^{2+}-free KRBH ($(-)Ca^{2+}$ KRBH) was made with the same composition as KRBH except NaCl was 142.25 mM, $CaCl_2$ was omitted, and 0.1 mM EGTA was added.

GH_4C_1 cells were grown in Ham's F-10 medium supplemented with 15% horse serum and 2.5% fetal calf serum (F-10^+) at 37°C for 4–5 days, both on glass coverslips (10^5 cells/slip) for monitoring $[Ca^{2+}]_i$ and in 60 mm Petri dishes with 10 mg sterile Cytodex-3 beads (2×10^6 cells/dish) for studying PRL secretion. Dynamics of $[Ca^{2+}]_i$ and PRL secretion were analyzed as reported previously (Sato et al., 1990d). Briefly, $[Ca^{2+}]_i$ was monitored by dual excitation microfluorimetry (FluoroPlex III fluorimeter, Tracor Northern, WI, U.S.A.) after loading cells with fura-2 by exposure to 2 μM fura-2/AM (Molecular Probes, Eugene, OR, U.S.A.) in KRBH for 30 min at 37°C. From the ratio ($r = F_{340}/F_{380}$) of the fluorescence excited at the two wave lengths, from which background was subtracted, $[Ca^{2+}]_i$ was calculated. Dynamics of PRL secretion were analyzed by column perifusion. Flow rate was 0.6 ml/min and fractions were collected every 30 s. Samples were frozen at −20°C until analysis for PRL by radioimmunoassay using protocols and specific reagents supplied by the National Hormone and Pituitary Program.

Cell viability as measured by trypan blue staining was > 95% at both the beginning and the end of the experiment. Medium osmolarity was increased in graded steps from 300 to 600 mOsm by the addition of sucrose and measured with an Advanced DigiMatic Osmometer Model 3D3 with range of 0–2000 mOsm and a sensitivity of 1 mOsm. BAY K 8644 (Calbiochem, La Jolla, CA, U.S.A.) was dissolved in ethanol, with a final concentration in medium of 0.01%. Medium containing 0.01% ethanol solvent had no detectable effect on either $[Ca^{2+}]_i$ or PRL secretion in these cells (Sato et al., 1990c).

Statistical analysis was made with Student's *t*-test, or with linear correlation. For the analysis of $[Ca^{2+}]_i$ data, the TRH-induced second phase rise in $[Ca^{2+}]_i$ was calculated as the mean of $[Ca^{2+}]_i$ values during the 1 min just before the end of TRH stimulation.

Results

Effect of $(-)Ca^{2+}$ medium on both $[Ca^{2+}]_i$ and PRL secretory dynamics in response to TRH

With 1.5 mM Ca^{2+} in KRBH, basal $[Ca^{2+}]_i$ was 175 ± 12 nM ($n = 6$, mean ± SE). 4 min exposure to 10 nM TRH caused two phases of increase in $[Ca^{2+}]_i$; an initial high-amplitude first phase burst (497 ± 23 nM) and a sustained low-amplitude second phase (285 ± 31 nM) (Fig. 1*A*). Pretreatment with $(-)Ca^{2+}$ KRBH for 5 min decreased basal $[Ca^{2+}]_i$ (143 ± 9 nM; $P < 0.05$, Student's t) and completely abolished the TRH-induced second phase rise in $[Ca^{2+}]_i$. However, the TRH-induced first phase rise in $[Ca^{2+}]_i$ was only 21 ± 4% decreased by removal of medium Ca^{2+}.

With 1.5 mM Ca^{2+} in KRBH, basal PRL secretion was 2.5 ± 0.2 ng/ml ($n = 6$). 4 min exposure to 10 nM TRH induced a high-amplitude PRL secretory burst (14.6 ± 1.8 ng/ml), which peaked within 2 min after stimulation, and slowly returned to basal level (Fig. 1*B*). With $(-)Ca^{2+}$

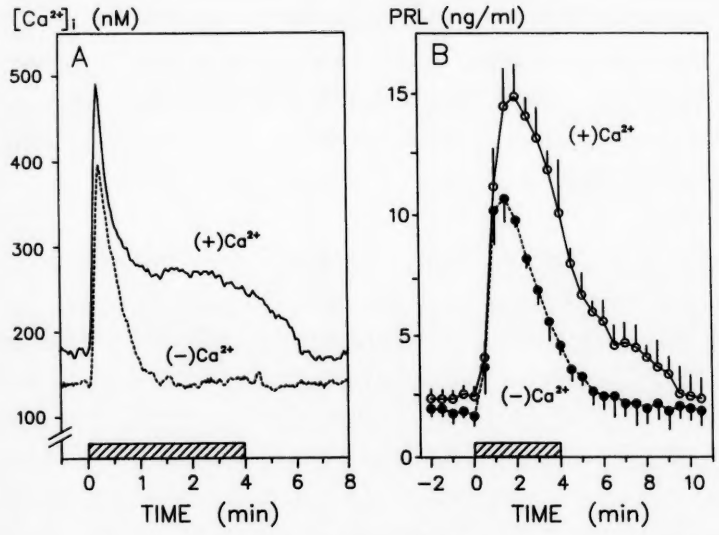

Fig. 1. Effect of medium Ca^{2+} on $[Ca^{2+}]_i$ and PRL secretory dynamics in GH_4C_1 cells in response to 10 nM TRH. The horizontal hatched bars indicate the duration of TRH stimulation. Experiments were performed as 'crossover' studies. Half of the cell preparations were initially studied with a normal Ca^{2+} concentration in the medium followed by the study without Ca^{2+} in medium; in the other half the order was reversed. *A*: Mean dynamics of $[Ca^{2+}]_i$ with 1.5 mM Ca^{2+} (solid line) or without Ca^{2+} (dashed line) in the medium, $n = 6$. *B*: Mean dynamics of PRL secretion with 1.5 mM Ca^{2+} (solid line, open circles) or without Ca^{2+} (dashed line, closed circles) in the medium. Mean ± SE of effluent PRL concentration in fractions collected every 30 s in three 'crossover' perifusion experiments.

KRBH, TRH caused a similar initial PRL secretory burst during the first minute of stimulation. PRL secretion peaked at 1.5 min after the onset of TRH stimulation with a peak PRL amplitude significantly less than that obtained with KRBH containing 1.5 mM Ca^{2+} ($P < 0.01$, Student's t).

Dose effect of medium hyperosmolarity on $[Ca^{2+}]_i$ dynamics in response to TRH

Medium hyperosmolarity transiently decreased basal $[Ca^{2+}]_i$ for the first 5 min of exposure (data not shown). Medium hyperosmolarity suppressed both the first and second phase rises in $[Ca^{2+}]_i$ caused by TRH in a dose-dependent manner (Figs. 2 and 3) ($r = -0.982$, $P < 0.001$, $n = 15$, and $r = -0.994$, $P < 0.001$, $n = 15$, respectively). However, in spite of only 20% suppression of first phase $[Ca^{2+}]_i$ rise by the highest degree of hyperosmolarity (600 mOsm), this degree of hyperosmolarity almost completely abolished the second phase rise in $[Ca^{2+}]_i$.

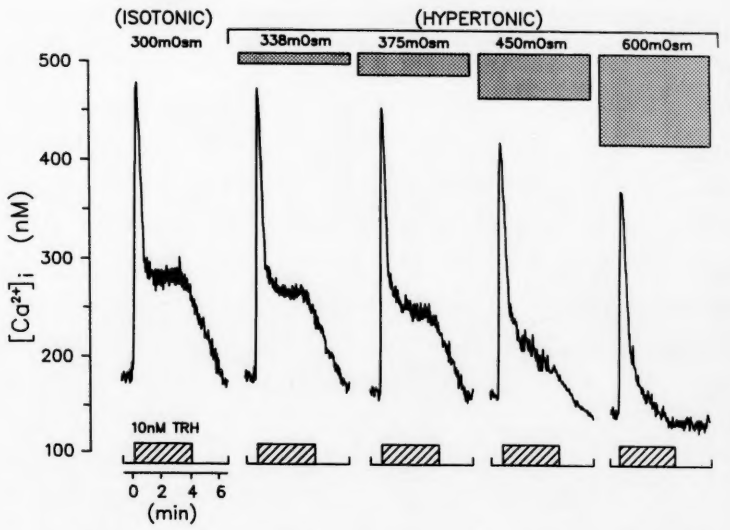

Fig. 2. Dose effect of medium hyperosmolarity on $[Ca^{2+}]_i$ dynamics induced by 10 nM TRH. GH_4C_1 cells were perfused with hypertonic medium (338, 375, 450, and 600 mOsm; 300 mOsm = isotonic) 5 min before TRH stimulation. Data are from a single experiment representative of three similar experiments in which the sequence of exposure with different doses of osmolarity was altered in each experiment.

Dose effect of medium hyperosmolarity on PRL secretory dynamics in response to TRH

Although hyperosmolarity did not significantly suppress basal PRL secretion, it depressed TRH-induced PRL secretion in a dose-dependent manner (Fig. 4). With 12.5, 25, or 50% hypertonicity (338, 375, and 450 mOsm, respectively), TRH-induced PRL secretion was suppressed especially after the first 1.5 min of TRH stimulation. However, the temporal initiation and ascending limb of PRL secretion by TRH stimulation was un-

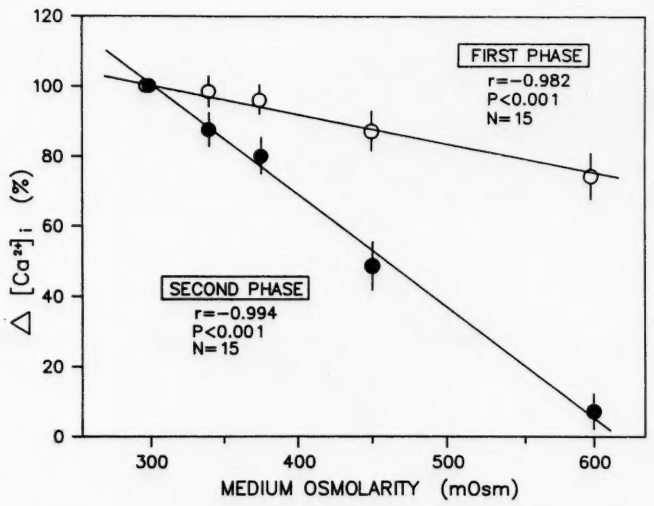

Fig. 3. Correlation of medium hyperosmolarity with the rise in $[Ca^{2+}]_i$ for initial high amplitude phase (first phase) and the following sustained (second) phase from the data in Fig. 2.

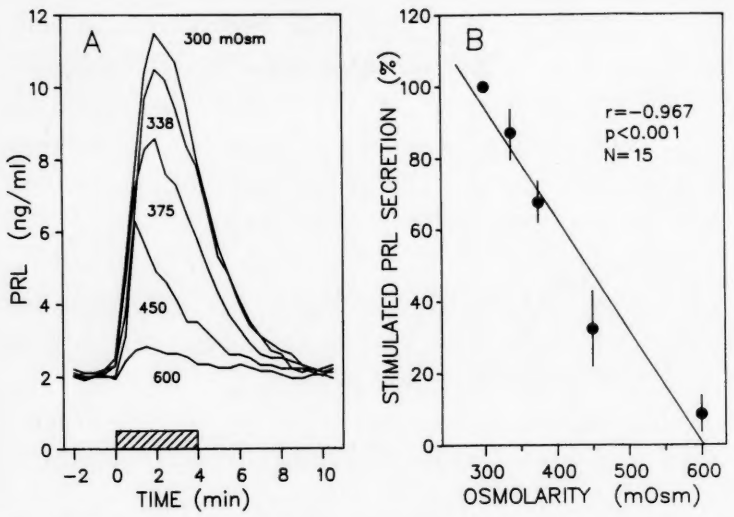

Fig. 4. Effect of graded degrees of medium hyperosmolarity on PRL secretion induced by 10 nM TRH. GH_4C_1 cells were perfused with hypertonic medium (338, 375, 450, and 600 mOsm; 300 mOsm = isotonic) 5 min before TRH stimulation. *A*: Dose effect of medium hyperosmolarity on PRL secretory dynamics. The horizontal hatched bar indicates the duration of TRH stimulation. The mean of three experiments is shown. The numbers indicate the osmolarity (mOsm). *B*: Correlation between hyperosmolarity and TRH-stimulated integral PRL secretion from the data in *A*, expressed as percent of PRL secretion in isotonic medium.

changed by even 450 mOsm hyperosmolarity (Fig. 4*A*). According to the data in this study demonstrating the effect of $(-)Ca^{2+}$ medium on both $[Ca^{2+}]_i$ and PRL secretion dynamics in response to TRH (Fig. 1), this initial high-amplitude burst of PRL secretion should be due to the preceding

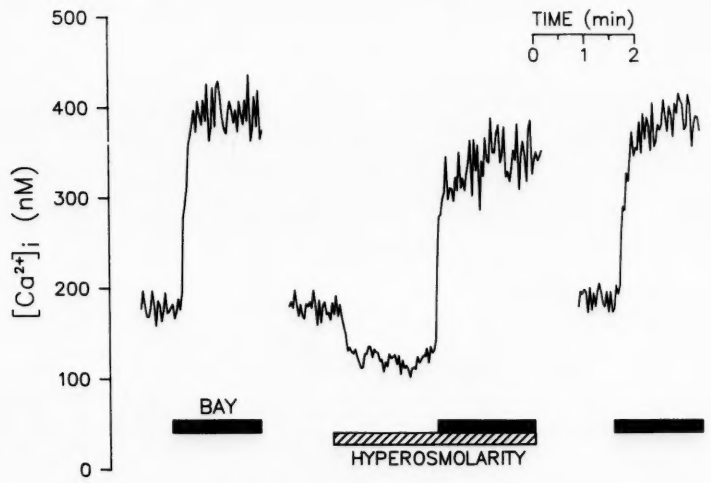

Fig. 5. Effect of hyperosmolarity on BAY K 8644-induced rise in $[Ca^{2+}]_i$. The horizontal closed bars and hatched bar indicate the duration of 1 μM BAY K 8644 (BAY) and 100% hyperosmolarity, respectively. Cells were perifused with normal KRBH for 15 min between each experiment. The data shown are representative of three similar experiments.

initial high-amplitude first phase rise in $[Ca^{2+}]_i$, which is mobilized from intracellular Ca^{2+} pools. However, 100% hyperosmolarity (600 mOsm) blocked the initial PRL secretory burst but not the $[Ca^{2+}]_i$ rise.

Effect of 100% hyperosmolarity on the BAY K 8644-induced rise in $[Ca^{2+}]_i$

BAY K 8644 is a specific dihydropyridine (DHP)-sensitive Ca^{2+}-channel agonist which increases $[Ca^{2+}]_i$ by inducing Ca^{2+} influx through these channels (Schramm et al., 1983). As shown in Fig. 5, 1 μM BAY K 8644 caused an acute increase in $[Ca^{2+}]_i$. However, 100% hyperosmolarity could not suppress this BAY K 8644-induced increased in $[Ca^{2+}]_i$.

Discussion

Previous in vitro studies have shown that extracellular hyperosmotic states inhibit exocytosis in many cells (Rink and Knight, 1988). This has been interpreted as a direct effect on membrane fusion during exocytosis. According to the chemosmotic hypothesis (Pollard et al., 1977, 1984), an accumulation of osmotically active particles within the secretory granule due to neutralization of the intragranular H^+ with extragranular anions, or exchange of H^+ with extragranular cations causes the granule to swell and facilitate membrane fusion. This induced osmotic force is involved in the regulation of the release of bovine PRL from secretory granules (Lorenson and Jacobs, 1987). Extracellular hyperosmolarity is believed to suppress this osmotic force and inhibit secretion (Holz, 1986; Rink and Knight, 1988). However, there are very limited data about the influence of extracellular hyperosmolarity on second messenger systems. We conclude from the results described in this report that in GH_4C_1 cells the inhibition of TRH-induced PRL secretion by medium hyperosmolarity, at least in part, depends on the suppression of extracellular Ca^{2+} influx in addition to an effect of hyperosmolarity on exocytosis through some other mechanism.

Removal of medium Ca^{2+} abolished the second phase rise in $[Ca^{2+}]_i$ in response to TRH, as previously described in GH_3 (Gershengorn, 1986) and GH_4C_1 cells (Tashjian, 1990), suggesting that

the second phase $[Ca^{2+}]_i$ increase is due to Ca^{2+} influx across the plasmalemma. Although it is not clear how this Ca^{2+} influx participates in PRL secretion, removal of medium Ca^{2+} suppressed both the second phase rise in $[Ca^{2+}]_i$ and PRL secretion after 1–1.5 min of TRH stimulation, suggesting that Ca^{2+} influx is an important factor in the later phase of TRH-stimulated PRL secretion. The effect of Ca^{2+} influx on secretion is probably coordinated with a diacylglycerol response (Gershengorn, 1986; Tashjian, 1990). In this study, medium hyperosmolarity suppressed both the second phase rise in $[Ca^{2+}]_i$ in response to TRH and PRL secretion after 1–1.5 min of TRH stimulation, suggesting that medium hyperosmolarity inhibits TRH-induced PRL secretion by suppressing Ca^{2+} influx through the plasmalemma.

There are at least two possible explanations of the suppression of Ca^{2+} influx by medium hyperosmolarity: (1) a direct physical effect on the Ca^{2+} channel itself, or (2) an indirect effect on Ca^{2+}-channel regulation. In our present study, 100% hyperosmolarity did not suppress the rise in $[Ca^{2+}]_i$ induced by BAY K 8644, which directly opens DHP-sensitive Ca^{2+} channels (Schramm et al., 1983), although it almost completely abolished the TRH-induced second phase $[Ca^{2+}]_i$ rise, most of which depends on second messenger and/or membrane depolarization-induced Ca^{2+} influx through DHP-sensitive Ca^{2+} channels (Tashjian, 1990). Thus, medium hyperosmolarity affects Ca^{2+}-channel regulation indirectly. For example, protein kinase C may be involved because: (1) protein kinase C is activated during volume changes in many cells (Grinstein et al., 1986); (2) pretreatment with phorbol esters (protein kinase C activators) inhibits Ca^{2+} influx through DHP-sensitive Ca^{2+} channels in response to specific agonists (Izumi et al., 1990); (3) pretreatment with phorbol esters suppressed the increase in both PRL secretion and $[Ca^{2+}]_i$ caused by cell swelling due to either hyposmolarity or permeant molecules (unpublished data); (4) depolarizing concentrations of K^+ stimulate PRL secretion by increasing Ca^{2+} influx through voltage-dependent Ca^{2+} channels which appear regulated by protein kinase C (Gershengorn, 1986). Increasing medium osmolarity up to 2-fold suppressed the increase in both $[Ca^{2+}]_i$ and PRL secretion in a dose-dependent manner (Wang, Sato, and Greer, unpublished). To delineate the mechanism in detail by which cell volume affects Ca^{2+} channels, further studies are required. The present data provide further evidence of our hypothesis that GH_4C_1 cells possess Ca^{2+} channels which are affected by cell volume (Sato et al., 1990b, d). Extracellular hyperosmolarity, which shrinks cell volume, closes Ca^{2+} channels in this study. Conversely, cell swelling induced by medium hyposmolarity or permeant molecules opens DHP-sensitive Ca^{2+} channels in these cells (Sato et al., 1990b, c, d).

Although our data indicate that suppression of Ca^{2+} influx is an important mechanism by which hyperosmolarity depresses TRH-induced PRL secretion, other factors are involved. Ca^{2+}-unrelated mechanisms, possibly direct suppression of osmotic forces which cause secretory vesicle swelling, membrane fusion, and dispersal of vesicle contents (e.g. hormone) (Zimmerberg et al., 1987), also play an important role. In our study a 2-fold increase in extracellular osmolarity almost completely abolished TRH-induced PRL secretion while only slightly depressing the first phase rise in $[Ca^{2+}]_i$. Although we did not evaluate the effect of hyperosmolarity on BAY K 8644-induced PRL secretion in this study, it is highly probable that high degrees of hyperosmolarity will suppress BAY K 8644-induced PRL secretion since hyperosmolarity suppresses events in the secretory transduction chain which occur after an increase in $[Ca^{2+}]_i$.

Hyperosmotic states occur frequently in some diseases, e.g. diabetes mellitus, dehydration. Our data indicate that an impaired Ca^{2+} signal may play a role in these conditions.

Acknowledgements

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Expression of transforming growth factor-α and epidermal growth factor receptor in human fetal kidneys

Paul R. Goodyer, Jehane Fata, Lois Mulligan, Daniel Fischer, Richard Fagan, Harvey J. Guyda and Cynthia G. Goodyer

Department of Pediatrics, McGill University – Montreal Children's Hospital Research Institute, and Ludwig Institute for Cancer Research, Montreal, Quebec, Canada

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Summary

Beginning at the fifth week of fetal life, successive generations of individual nephrons are induced by contact between metanephric mesenchyme and ureteric bud. Following phenotypic transformation, cells of each primitive renal vesicle undergo a phase of rapid cell division. In order to identify genes which might regulate nephron development in man, we screened adult and fetal kidney RNA for expression of a panel of growth-related genes. Among the genes which were expressed at higher levels in fetal kidney was the epidermal growth factor (EGF) receptor. There is controversy as to the most likely physiologic EGF receptor ligand in fetal kidney; we were able to identify a transcript for transforming growth factor-α (TGF-α) but not EGF on Northern blots of fetal kidney RNA. Since the abundance of TGF-α mRNA is low, we confirmed its presence by polymerase chain reaction amplification. Using specific radioimmunoassays, we also provide direct evidence for TGF-α but not EGF peptide in extracts of fetal kidney and mid-gestational amniotic fluid. We suggest that TGF-α/EGF receptor interactions may serve an important function in development of human fetal kidney.

Introduction

Between the fifth and sixth weeks of human fetal life, undifferentiated metanephric mesenchyme is contacted by the ureteric bud (an outpouching of the Wolffian duct) resulting in induction of the first generation of nephrons (Bacallao and Fine, 1989). Prior to induction, the metanephric tissue consists of loosely organized cells proliferating in an extracellular matrix of fibronectin and collagens type I and III. Histologic observations indicate that clusters of metanephric cells condense at the point of contact with individual branches of the ureteric bud and then vesiculate to form S-shaped tubular structures (Pritchard-Jones et al., 1990). This conversion of mesenchyme to epithelium involves transient expression of critical transcription factors such as N-myc (Mugrauer et al., 1988) and Wilms' tumour gene (Pritchard-Jones), and a shift from synthesis of interstitial collagens to basement membrane-specific components such as type IV

Address for correspondence: Dr. Paul R. Goodyer, Division of Pediatric Nephrology, Montreal Children's Hospital, 2300 Tupper Street, Montreal, Quebec H3H 1P3, Canada. Tel. (514) 934-4461; Fax (514) 934-4477.

collagen and laminin (Ekblom et al., 1981, 1990). The cells become polarized and exhibit increased lateral adhesion (Klein et al., 1988).

Induction of metanephric mesenchyme causes a shift in cellular phenotype, but it also initiates a phase of rapid cell division. Little is known about the mitogenic signals which direct this proliferation, but it is clear that local regulatory events are involved. Selective cell division and ingrowth of capillaries at one end of the elongating renal vesicle allow formation of a primitive vesicle; proliferation at its distal end achieves fusion with ureteric bud and access to the bladder.

In this study, we screened a panel of human adult and fetal kidney RNAs with 21 growth-related cDNA probes in order to identify molecular mechanisms potentially involved in fetal kidney growth. Among the genes selectively expressed at high levels in fetal samples was that for the epidermal growth factor (EGF) receptor. Additional studies establish transforming growth factor-α (TGF-α) as the most likely endogenous ligand for fetal kidney EGF receptor.

Materials and methods

Tissues and materials

Human fetal kidneys (8–20 weeks fetal age) and placenta (18 weeks gestational age) were obtained with informed consent and approval of local hospital ethics committees at the time of therapeutic abortion. Normal postnatal kidney was obtained from adults ($n = 4$) and a 4-year-old girl at the time of nephrectomy for well-demarcated renal tumours. The tissues were rapidly frozen and stored at $-70\,^{\circ}C$ for later use. Aliquots of amniotic fluid were obtained with informed consent from pregnant women undergoing diagnostic amniocentesis between 14 and 40 weeks gestation.

Human EGF was obtained from Collaborative Research (Bedford, MA, U.S.A.), [^{125}I]sodium iodide from Amersham Canada (Oakville, Ont., Canada), and chloramine T and other reagents were obtained from Fisher Scientific Co. (Fair Lawn, NJ, U.S.A.) or Sigma Chemical Co. (St. Louis, MO, U.S.A.). Restriction enzymes and other molecular biology reagents were purchased from Bethesda Research Laboratories (Gaithersburg, MD, U.S.A.).

RNA isolation and analysis

For Northern analyses, frozen tissues were pulverized on dry ice; total RNA was prepared by the guanidinium/cesium chloride gradient method (Chirgwin et al., 1979). 30 μg of total RNA was separated on 1% agarose/formaldehyde gels and transferred to nitrocellulose or Hybond-N membranes (Amersham) using 10 × SSC (1.5 M sodium chloride, 0.15 M sodium citrate, pH 7.0). The Northern blots were hybridized for 36 h to ^{32}P-labelled probes (random oligonucleotide priming) in 50% formamide, 40 mM Na_2HPO_4 (pH = 6.7), 5 × SSC, 0.05% sodium dodecyl sulphate (SDS), 0.02% polyvinylpyrrolidone (PVP), 0.02% Ficoll and 0.02% bovine serum albumin at 42 °C. The blots were washed at low stringency and then at 50 °C for 20 min (0.1 × SSC, 0.1% SDS) prior to autoradiography.

Reverse transcriptase / polymerase chain reaction

5 μg of total RNA was mixed with reverse transcriptase (RT) buffer (Bethesda Research Laboratories), 20 U of RNAase inhibitor (Promega) and 5 μl of antisense 3′ primer (5′..AGA ATG GCA GAC ACA TGC TGG..3′) (10 μM) synthesized for a sequence spanning an exon–exon splice site (amino acids 69–75) in the mature human TGF-α cDNA (Derynck et al., 1984); total volume = 16.5 μl. After heating to 65 °C for 5 min, the mix was chilled and combined with 1 μl (200 U) reverse transcriptase (Bethesda Research Laboratories) and 2.5 μl dNTP mix (4 mM each), and incubated for 45 min at 37 °C.

For polymerase chain reaction (PCR) amplification, the RT product was combined with 5 μl dNTPs (4 mM each), 10 μl of 10 × Perkin-Elmer PCR buffer, 0.5 μl (2.5 U) Taq polymerase (Bethesda Research Laboratories), 44.5 μl H_2O, 10 μl of the 3′ primer (10 μM) and 10 μl of a sense 5′ oligonucleotide primer (5′..TTC GCT CTG GGT ATT GTG TTG..3′) synthesized from a sequence within the putative signal sequence (amino acids 2–8) for human TGF-α (Derynck et al., 1984). The mix was covered with a layer of mineral oil and submitted to 20 or 25 PCR cycles (94 °C × 1 min, 55 °C × 1.5 min, 72 °C × 2 min) in a Perkin-Elmer thermocycler. Nitrocellulose Southern blots of PCR products were hybridized (5 × SSPE (0.75 M NaCl, 50 mM NaH_2PO_4, 5 mM EDTA-Na_2,

pH 7.4) 0.5% SDS, 5 × Denhardt's, 65°C) to a ^{32}P-end-labelled oligonucleotide probe (5′..ACG GGT CTA AGG GTG TGA GTC..3′) corresponding to a human TGF-α sequence midway (amino acids 50–58) between the amplification primers. The blots were washed and analyzed by autoradiography.

Radioimmunoassays

For assay of transforming growth factor-α, frozen kidney was pulverized on dry ice and homogenized in 5 volumes of buffer containing 20 mM Tris (pH 7.4), 0.25 M sucrose, 1 mM phenylmethylsulfonyl fluoride, and 0.1% Triton X-100. TGF-α was measured in amniotic fluid and tissue extracts in triplicate with the Biotope TGF-α radioimmunoassay (RIA) kit (Biotope, Redmond, WA, U.S.A.). The kit utilizes antiserum raised against synthetic TGF-α peptide (17 residues, 34–50) and gives linear displacement for human TGF-α standard between 0.5 and 10 pmol/ml but does not recognize human EGF. Half-maximal competition is at 1.4 nM TGF-α. Serial dilutions of kidney extracts or amniotic fluid samples produced displacement of TGF-α tracer parallel to the standard curve; Triton X-100 buffer gave no displacement in the assay.

For assay of human EGF, tissues were prepared as above. EGF was measured by radioimmunoassay as described previously (Goodyer et al., 1990). The antiserum used was raised in rabbits against recombinant human EGF (Amgen, Boston, MA, U.S.A.); it detects human EGF but not human or rat TGF-α, nor mouse or rat EGF. Half-maximal competition is 0.38 nM. Serial dilutions of kidney extracts or amniotic fluid produced displacement parallel to the standard curve; Triton X-100 buffer gave no displacement in the assay.

Specific high-affinity EGF binding was performed as previously described (Goodyer et al., 1988). Frozen tissues were homogenized in 5 volumes of buffer containing 20 mM Tris (pH 7.4) and 0.25 M sucrose. The homogenate was centrifuged twice at 500 × *g*, and the supernatant respun at 20,000 × *g* for 20 min to obtain a membrane pellet. Human EGF was iodinated by the chloramine T method and incubated (50,000 cpm/assay) with 200–300 μg kidney membrane protein or 100 μg placental membrane protein in 500 μl final volume of 25 mM Tris (pH 7.4)/10 mM magnesium chloride buffer containing 1 mg/ml bovine serum albumin and 1 mg/ml bacitracin at 4°C for 18 h. Specific binding was estimated by comparing the fraction of total tracer bound in the presence or absence of excess unlabelled EGF (500 ng/ml).

Results

Gene expression in fetal and adult kidney

The expression profile of 21 genes suspected of involvement in regulation of cell growth was assessed by Northern analysis, using a panel of total RNA samples (30 μg per lane) from human fetal ($n = 11$) and adult kidneys ($n = 4$). Fetal foot length was used to determine fetal age; samples ranged from 12 to 22 weeks fetal age. For simplicity, semi-quantitative estimates of signal intensity for each gene were assigned to early (12–18 weeks) and mid (19–22 weeks) gestation fetal kidney samples and to the adult panel.

Five of the 21 genes studied (NGF, IGF-I, IL-2, c-abl, and L-myc) were undetectable by Northern analysis in any of the fetal or adult kidney samples. Transcripts for ten of the 21 genes (c-myc, c-raf, c-neu, TGF-β1, c-met, c-yes, c-sis, RB1, K-ras, H-ras) were detected in all samples, but the level of gene expression was equivalent in the early fetal, mid-fetal and adult kidney groups. The expression pattern for the remaining six genes is summarized in Table 1. These included three transcription factor genes (N-myc, c-myb and c-erbA), a homebox gene (HHO.c13), a growth factor gene (IGF-II) and a growth factor receptor gene (EGF receptor). In each case, transcript was detected in early and mid-fetal kidney

TABLE 1

EXPRESSION OF GROWTH-RELATED GENES IN HUMAN KIDNEY

Gene	Fetal kidney (12–22 weeks)	Adult kidney
IGFII	++++	−
N-myc	++	−
c-myb	++	−
c-erbA	+++	+/−
HHO.c13	+	−
EGF-R	++	+/−

but was much reduced or absent in adult kidney. Expression of EGF receptor and its potential ligands were analyzed in detail.

EGF receptor

To analyze EGF receptor transcript heterogeneity, Northern blots of 18-week fetal kidney and placenta were probed with the human EGF receptor cDNA (American Type Culture Collection). We noted two primary transcripts (9.4/5.6 kb) which hybridize to the probe in both tissues (Fig. 1*a*). These transcripts correspond to the mRNA banding pattern described in other human tissues (Xu et al., 1984) suggesting that there is no unusual EGF receptor mRNA processing in fetal kidney. The steady-state level of each fetal kidney EGF receptor transcript is considerably less than in placenta, about 1 : 8–10 when the slight difference in ethidium bromide-stained RNA in each lane is taken into account. The blot was also probed with a β-actin cDNA (Fig. 1*b*), but expression of this gene does not appear to be constant during development. Expression of EGF receptor mRNA in normal kidney from a 4-year-old child was barely detectable by Northern analysis (data not shown).

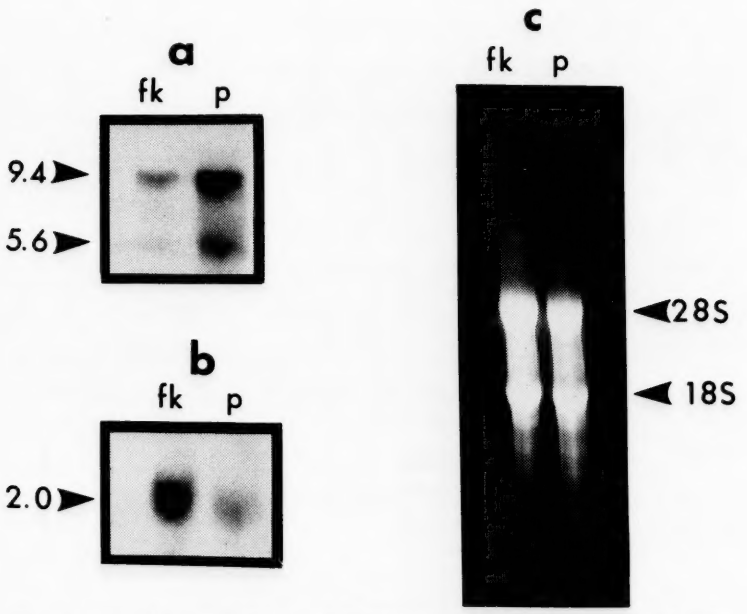

Fig. 1. Northern analysis of EGF receptor mRNA. (*a*) Northern analysis of total RNA from 18-week fetal age human kidney (lane fk, 20 μg) and mid-term placenta (lane p, 10 μg): two primary transcripts are noted (9.4 and 5.6 kb) which hybridize to the human EGF receptor probe. (*b*) The same Northern blot was stripped and re-probed for β-actin. (*c*) The ethidium bromide-stained gel demonstrates integrity of RNA in all samples.

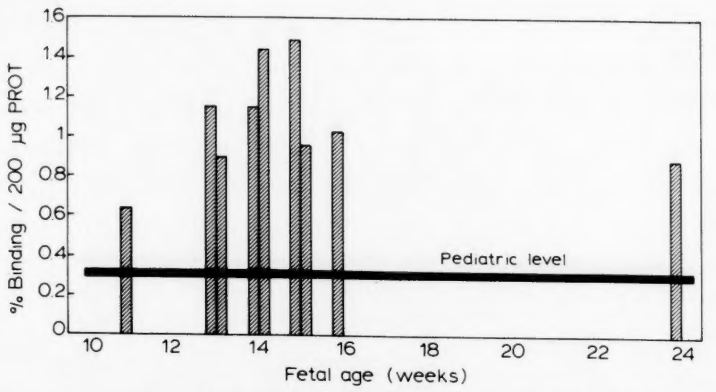

Fig. 2. Specific high-affinity EGF binding. Iodinated human EGF was incubated (18 h on ice) with kidney membranes sedimenting between 1000 and 20,000 $\times$ *g*. Total specific binding (total cpm bound/200 μg protein minus cpm bound in the presence of excess unlabelled EGF) is expressed as a percentage of total cpm in the assay (mean of four replicates). Each bar represents an individual pooled pair of kidneys. Pediatric level on normal kidney from a 4-year-old girl is the mean of three separate determinations.

Specific high-affinity EGF binding (Fig. 2) was measured in membrane fractions of placenta, fetal kidney and normal kidney from a 4-year-old child. In mid-gestational samples, EGF binding (0.65–1.5% cpm bound/200 μg protein) was 2–5 times higher than in the postnatal kidney (0.35 $\pm$ 0.05% (SEM) of total cpm bound/200 μg protein), but was less than in mid-gestational placenta (27.9 $\pm$ 2.4% cpm bound/200 μg protein).

TGF-α and EGF in fetal kidney

If the EGF receptor is to influence fetal kidney growth, it must be activated by an appropriate mitogenic ligand. To compare the levels of TGF-α and EGF peptides present in fetal kidney, snap-frozen tissues were extracted into Tris buffer containing Triton X-100 (0.1%) for radioimmunoassay. As seen in Fig. 3, TGF-α was detectable (35–45 pmol/g wet weight) by specific radioimmunoassay in all fetal kidneys from 11–15 weeks gestation. In one pair of kidneys obtained at 7–8 weeks gestation, the level was significant but was considerably lower.

Mid-gestational kidney extracts were also assessed for EGF content by specific radioimmunoassay. In all fetal kidney samples, EGF was undetectable (lower limit of assay = 0.1 pmol/ml). By contrast, the extract from normal kidney of a 4-year-old girl contained 6 pmol/g wet weight.

Since renal EGF apparently undergoes luminal

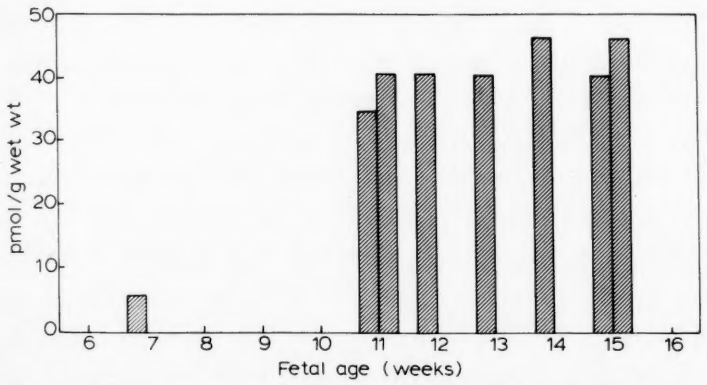

Fig. 3. TGF-α peptide in human fetal kidney. Eight human fetal kidney samples (fetal age 7–15 weeks) were extracted with Tris buffer containing Triton X-100 (0.1%) for radioimmunoassay of TGF-α.

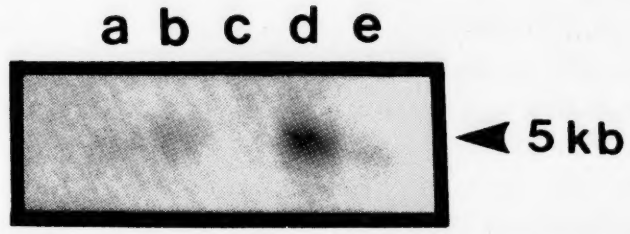

Fig. 5. Northern analysis of TGF-α mRNA in human fetal kidney. Northern blots of total RNA from pediatric (4-year-old female) liver (lane a), 18-week fetal age kidney (lane b = 30 μg, lane c = 15 μg), and pediatric kidney (4-year-old female) (lane d = 30 μg, lane e = 15 μg) were probed with a labelled cDNA for human TGF-α. A single 5 kb transcript is seen in lanes b and d, but is below the limit of detection in lanes a and c, and is only faintly detectable in lane e.

cleavage and urinary excretion in postnatal life, we assayed the levels of EGF in amniotic fluid at various gestational ages (15–40 weeks) to determine whether EGF might be produced by the fetal kidney. TGF-α levels also were measured in the same samples. As seen in Fig. 4, amniotic fluid EGF is barely above the limit of reliable detection until 30 weeks gestation, and then rises slowly, reaching 0.1–0.2 nM just prior to birth. However, TGF-α is measurable at much higher levels in all amniotic fluid samples (2.5–7.5 nM). Even in term samples, the concentration (4.5 nM) is about 35 times that of EGF (0.13 nM). There is some suggestion that the level of amniotic TGF-α may be biphasic, with a major peak at 20–25 weeks gestation, and a second peak at 35–40 weeks.

To ascertain whether kidney TGF-α is produced locally, we analyzed total RNA from fetal and pediatric kidney. Northern analysis demonstrates a single 5 kb transcript in both the fetal

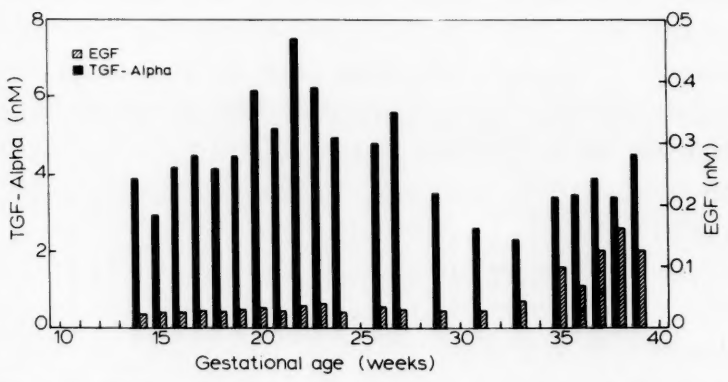

Fig. 4. EGF and TGF-α peptides in human amniotic fluid. Undiluted samples of human amniotic fluid were analyzed for EGF and TGF-α using specific radioimmunoassays. Each bar represents the mean of 1–6 samples. TGF-α levels appear to peak at 20–25 weeks, fall slightly, and rise again just prior to term. EGF levels are not significantly above the limit of detection until 30–35 weeks gestation, when they begin to rise. Note that at term, EGF (0.1–0.2 nM) is only 2–4% of the concentration of TGF-α (3.5–4.5 nM).

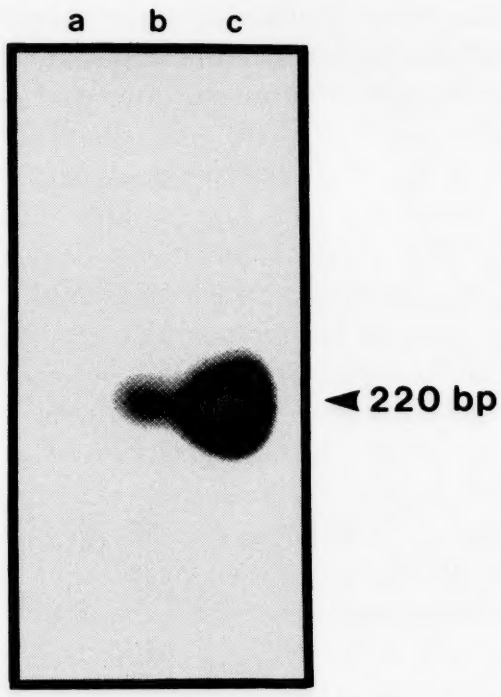

Fig. 6. Southern analysis of TGF-α PCR products from fetal kidney RNA. Total RNA from human 18-week fetal age kidney was submitted to reverse transcriptase reaction followed by 20 (lane b) or 25 (lane c) polymerase chain reaction cycles using oligonucleotide primers for a human TGF-α coding sequence. Southern blots of the PCR products were hybridized to a labelled oligonucleotide probe corresponding to a 22 base sequence midway between the primers. The predicted single 220 base fragment is identified (lanes b and c). No band is seen (lane a) when reverse transcriptase is omitted from the first step.

and pediatric kidney samples when 30 μg of total RNA are loaded, but the signal is barely detectable at 15 μg/lane (Fig. 5). To confirm that there is TGF-α expression in the fetal sample, 5 μg of total RNA was amplified by PCR as outlined in Materials and Methods. Southern blots of the PCR product were probed with a labelled 22-mer oligonucleotide for TGF-α (Fig. 6). A single band of expected PCR fragment size (220 bases) is identified; band intensity increases with cycle number (20 cycles, lane b vs. 25 cycles, lane c). The band does not represent amplification of genomic DNA since primers were designed to span exon–exon junctions and no signal is seen if reverse transcriptase is omitted from the RT step.

Discussion

In this report, we examine the expression profile of a panel of growth-related genes in an attempt to identify those which may be of particular importance to human fetal kidney development. Steady-state mRNA levels in early to mid-gestational fetal kidney were contrasted to those in adult renal tissue; several patterns warrant comment.

As noted by other investigators (Gray et al., 1987; Brice et al., 1989), we found insulin-like growth factor-II (IGF-II) mRNA to be abundant in human fetal kidney between 10 and 24 weeks of gestation, but nearly undetectable (by Northern analysis) in the adult. By contrast, we were unable to detect IGF-I mRNA in our fetal kidney panel; Han et al. (1987) have reported low levels in human fetal kidney by in situ hybridization. The importance of IGF-II as a fetal growth factor relative to IGF-I is not yet clear but transgenic mouse pups heterozygous for a disrupted IGF-II gene are only 60% the size of their wild-type littermates (DeChiara et al., 1990).

We found that levels of N-myc were high in mid-gestational human kidney, especially in the earliest period studied (12–18 weeks fetal age); the gene is not expressed or is expressed at very low levels in mature tissue. In the mouse, a similar pattern has been reported (Zimmerman et al., 1986). Fetal mouse kidney N-myc expression is apparently confined to metanephric cells during very early stages of differentiation and falls abruptly at the onset of overt terminal differentiation (Ekblom et al., 1981). In other rapidly dividing cells derived from ureteric bud, expression was also low. It was argued, therefore, that N-myc is not simply a marker of rapid cell division but a regulator of genes involved in early differentiation (Ekblom et al., 1981). HHO.c13, a human homeobox gene (Mavilio et al., 1986) may also fall into this category. We found that it was detectable in fetal human kidney throughout mid-gestation but not in the adult. Mammalian analogs such as the mouse HOX 1.1 gene are transiently expressed at high levels along the neural tube and at lower levels in 12.5-day fetal mouse kidney (Jackson et al., 1985; Mahon et al., 1988); while these genes are clearly involved in cellular pattern formation in insect development, their function in mammalian systems is not yet clear.

Thyroxine receptor mRNA, detected by hybridization to the c-erbA probe, was especially elevated in the later group of fetal kidneys (19–22 weeks fetal age). Since many renal enzyme systems are induced by thyroxine in the last trimester of fetal life, c-erbA may be involved in late events of terminal differentiation.

Certain proto-oncogenes were not detected in fetal kidney. Expression of these genes may not be critical to renal development, but it is possible that mRNA levels below the limits of detection by Northern analysis might exert significant influence on growth. Other genes such as TGF-β were expressed in fetal kidney, but not above the level in adult tissue. An important developmental function cannot be ruled out since the mRNA may be regulated post-transcriptionally, peptide levels may be controlled through post-translational cleavage, or peptide action might be limited by receptor availability.

EGF receptor mRNA levels were greater in fetal (16–18 weeks fetal age) than in adult kidney; this was in keeping with an increase in high-affinity EGF binding to fetal kidney membranes. However, if the EGF receptor is involved in fetal kidney growth, the source of its physiologic ligand has not been entirely clear. EGF is abundant in mature mammalian kidney and urine, but levels in human pre-term amniotic fluid (Scott et al., 1989) are low; urinary EGF rises gradually in the postnatal period (Matilla, 1988). In mouse kidney,

EGF mRNA transcripts cannot be detected by Northern analysis until 2 weeks after birth (Popliker et al., 1987). Furthermore, EGF appears to be highly polarized to the luminal membrane of the distal tubule (Salido et al., 1986), whereas renal EGF receptors are localized to the basolateral membrane throughout the nephron (Goodyer et al., 1988).

There has been controversy about whether fetal kidney can produce an alternate physiologic ligand for the EGF receptor. Nexo et al. (1980) detected significant levels of an EGF-like material by receptor assay in extracts of mid-gestational mouse kidney which could not be accounted for by radioimmunoassayable EGF. Wilcox and Derynck (1988) reported transient TGF-α mRNA in fetal mouse kidney, but Han et al. (1987) could not detect a TGF-α transcript on Northern blots of fetal rat kidney RNA at 8 days gestation. TGF-α mRNA was reported in normal human adult kidney adjacent to renal tumours (Gomella et al., 1989), but others have been unable to confirm this (Mydlo et al., 1989).

In this report, we demonstrate a single TGF-α mRNA transcript in both human fetal (18 weeks fetal age) and pediatric (age 4 years) kidney. However, the level of TGF-α mRNA is close to the lower limit of detection by Northern analysis and may account for discrepancies in the literature. We confirmed the presence of TGF-α mRNA in fetal kidney by amplifying the mRNA with PCR. Using a specific radioimmunoassay, we also provide direct evidence for significant levels of TGF-α peptide in human fetal kidney and amniotic fluid. Conversely, immunoreactive EGF was barely detectable in amniotic fluid before 20 weeks gestation. Even in the last trimester, as amniotic EGF began to rise, TGF-α was by far the more abundant of the two peptides. The abundance of both TGF-α and EGF receptor in human fetal kidney suggests a significant role for this growth factor system in normal renal development.

Acknowledgements

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Pachytene spermatocytes regulate the secretion of Sertoli cell protein(s) which stimulate Leydig cell steroidogenesis

Makoto Onoda, Daniel Djakiew and Vassilios Papadopoulos

Department of Anatomy and Cell Biology, Georgetown University School of Medicine, Washington, DC 20007, U.S.A.

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Summary

The influence of germ cells (pachytene spermatocytes and round spermatids) on the secretion by Sertoli cells of the proteinaceous factor(s) which stimulates Leydig cell steroid biosynthesis was investigated. Sertoli cells from immature rats were cultured on plastic dishes or on Millipore filters impregnated with reconstituted basement membrane in bicameral chambers. Immature rat Sertoli cell secreted proteins (rSCSP; MW > 10,000), from conventional cultures, stimulated 4- to 5-fold steroid biosynthesis in normal rat and MA-10 mouse tumor Leydig cells, respectively. MA-10 cells were then used as a bioassay system for most studies, although purified rat Leydig cells were used in some cases to further confirm results obtained with MA-10 cells. rSCSP collected from both the apical and basal compartment of the chambers were examined for their ability to stimulate Leydig cell steroidogenesis. The Leydig cell stimulatory activity from Sertoli cells was found to be secreted in a polarized manner, with 80% of the total bioactivity found in the basal rSCSP. Addition of pachytene spermatocyte proteins (PSP) in the apical compartment of the chambers inhibited, in a time- and concentration-dependent manner, the basally directed Sertoli cell secretion of the Leydig cell stimulatory protein(s) by 85%. Similar results were obtained when freshly isolated pachytene spermatocytes were directly added on top of Sertoli cell epithelial sheets in the apical compartment of the chambers. In contrast, round spermatid proteins (RSP) did not exhibit a comparable effect to that of PSP in regulating the Sertoli cell secretion of the Leydig cell stimulatory activity. These results demonstrate that the Sertoli cell secreted protein(s) which stimulates Leydig cell steroid biosynthesis is secreted in a basally polarized direction, and its secretion is specifically modulated by pachytene spermatocytes.

Address for correspondence: Dr. Vassilios Papadopoulos, Department of Anatomy and Cell Biology, Georgetown University School of Medicine, 3900 Reservoir Road, NW, Washington, DC 20007, U.S.A.

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Introduction

In 1978, Aoki and Fawcett observed that locally produced atrophy of seminiferous tubules, with antispermatogenic drugs, led to an hypertrophy of the adjacent Leydig cells. This original

work has been confirmed and extended in various models of experimental disruption of spermatogenesis in vivo, which showed that the seminiferous tubules influence the neighboring Leydig cells (reviewed by De Kretser, 1987; Saez et al., 1987). The principal changes in the Leydig cells were an hypertrophy, an increased content of cellular organelles, and an increase in the steroidogenic capacity. Moreover, these studies indicated that the changes in the Leydig cells must be due to local factors originating from the seminiferous tubules rather than circulating substances such as luteinizing hormone (LH) (Risbridger et al., 1981).

In vitro studies, using segments of seminiferous tubules, confirmed the regulatory effect of seminiferous tubule secreted factors on Leydig cell steroidogenesis (Parvinen et al., 1984; Syed et al., 1985; Papadopoulos et al., 1986, 1987; Vihko and Huhtaniemi, 1989). It was also established that the cellular origin of this seminiferous factor(s) is the Sertoli cell (Janecki et al., 1985; Verhoeven and Cailleau, 1986, 1990; Papadopoulos et al., 1986, 1987; Saez et al., 1987). The secretion of this Sertoli cell protein(s), which stimulates Leydig cell steroidogenesis, appears to be under the control of germ cells present in each segment of the seminiferous tubules (Parvinen et al., 1984; Syed et al., 1985; Papadopoulos et al., 1987; Kamtchouing et al., 1988; Vihko and Huhtaniemi, 1989). However, direct evidence of the regulatory role of germ cells on the secretion of this Sertoli cell factor(s) remains to be demonstrated.

In order to examine in vitro the interrelation between germ cells and Sertoli cells (seminiferous epithelial compartment of the testis) and their effect on Leydig cell function (interstitial compartment) in an environment which more closely mimics that which occurs in vivo, we used the bicameral culture chamber system developed in this laboratory (Byers et al., 1986) with the modifications recently described by Onoda et al. (1990).

Materials and methods

General materials

Sources and characteristics of chemicals and other materials used in these studies have been previously described (Ojeifo et al., 1990; Onoda et al., 1990; Onoda and Djakiew, 1990, 1991; Papadopoulos et al., 1990), unless otherwise stated.

Sertoli cell isolation and culture

Sertoli cells were isolated from 18-day-old Sprague-Dawley rats by a three-step sequential enzyme digestion procedure, as previously described (Onoda et al., 1990). Sertoli cells were plated at 2.4×10^6 cells/0.64 cm^2/apical well in serum-free defined media (Onoda et al., 1990) on Millipore filters impregnated with reconstituted basement membrane (Matrigel) in bicameral chambers (Millicell-HA). After 3 days in culture contaminating germ cells were lysed in 20 mM Tris-HCl (pH 7.5) according to the method of Galdieri et al. (1981). Subsequently, the Sertoli cell cultures were maintained in serum-free defined media for up to an additional 5 days. Only those cultures which did not exhibit hydrodynamic equilibration of media between the apical and basal compartments of the bicameral chambers were deemed suitable for use in the following studies (Onoda et al., 1990). Apical and basal Sertoli cell conditioned media from each individual chamber were separately collected, centrifuged ($1500 \times g$ for 10 min at 4°C), concentrated/dialysed against 10 mM phosphate buffer (pH 7.4), using Centricon-10 concentrators (molecular weight cut-off 10,000; Amicon), and immediately tested for bioactivity in regulating Leydig cell steroidogenesis. In some experiments Sertoli cells were directly plated onto plastic dishes and cultured at 34°C for 10 days in serum-free defined media. Medium was changed every 2 days and collected from day 4 to 10. Sertoli cell conditioned media were centrifuged ($1500 \times g$ for 10 min at 4°C) and stored at −20°C. Before use the medium was concentrated/dialysed against 10 mM phosphate buffer (pH 7.4), using Centriprep-10 concentrators (molecular weight cut-off 10,000; Amicon).

Serum-free defined medium consisted of Dulbecco's modified Eagle's medium (DMEM) supplemented with 100 U/ml penicillin, 100 μg/ml streptomycin, 0.25 μg/ml fungizone, 100 ng/ml follicle-stimulating hormone (FSH), 2 μg/ml insulin, 10 ng/ml epidermal growth factor, 5 μg/ml human transferrin, 50 ng/ml vitamin A, 200 ng/ml vitamin E, 10^{-9} M hydrocortisone,

10^{-7} M testosterone, 10^{-8} M estradiol, 2 mM glutamine, 5 ng/ml sodium selenate, 1 mM sodium pyruvate, 22 mM sodium lactate, and 3 μg/ml cytosine arabinoside.

Isolation and culture of germ cells

Pachytene spermatocytes (PS) and round spermatids (RS) were isolated from 90-day-old Sprague-Dawley rats by centrifugal elutriation (Onoda and Djakiew, 1990, 1991) as modified from Shaper et al. (1990) and Meistrich et al. (1981). In brief, after a sequential enzyme digestion of the seminiferous tubules, a single cell suspension of seminiferous epithelium was loaded into the elutriator. PS with purities in excess of 90% eluted at a rotor speed of 2000 rpm, and a flow rate between 31 ml/min to 36 ml/min. RS with purities in excess of 90% eluted at a rotor speed of 2500 rpm and a flow rate of 25.25 ml/min. PS and RS were separately incubated in germ cell culture media (Ham's F12/DMEM containing 100 nM testosterone, 10 mM sodium lactate, 0.7 mM sodium pyruvate, and 2 mM L-glutamine) at a density of 1×10^6 cells/ml, and cultured for 3 days at 34°C to obtain conditioned media. Culture media were replaced every day, pooled, and stored at −20°C. The viability, as indicated by trypan blue exclusion, of PS in germ cell incubation media declined from 44% after 1 day to 4% after 3 days of culture (Onoda and Djakiew, 1991) during the preparation of PSP. The viability of RS in germ cell incubation media declined from 23% after 1 day to 4% after 3 days of culture. The conditioned media were concentrated/dialysed against ice-cold distilled water by centrifugation on Centriprep-10 concentrators and subsequently lyophilized. PS, PSP, or round spermatid protein (RSP) were then added on top of the Sertoli cell layer (apical compartment) cultured in bicameral chambers as shown in Fig. 1.

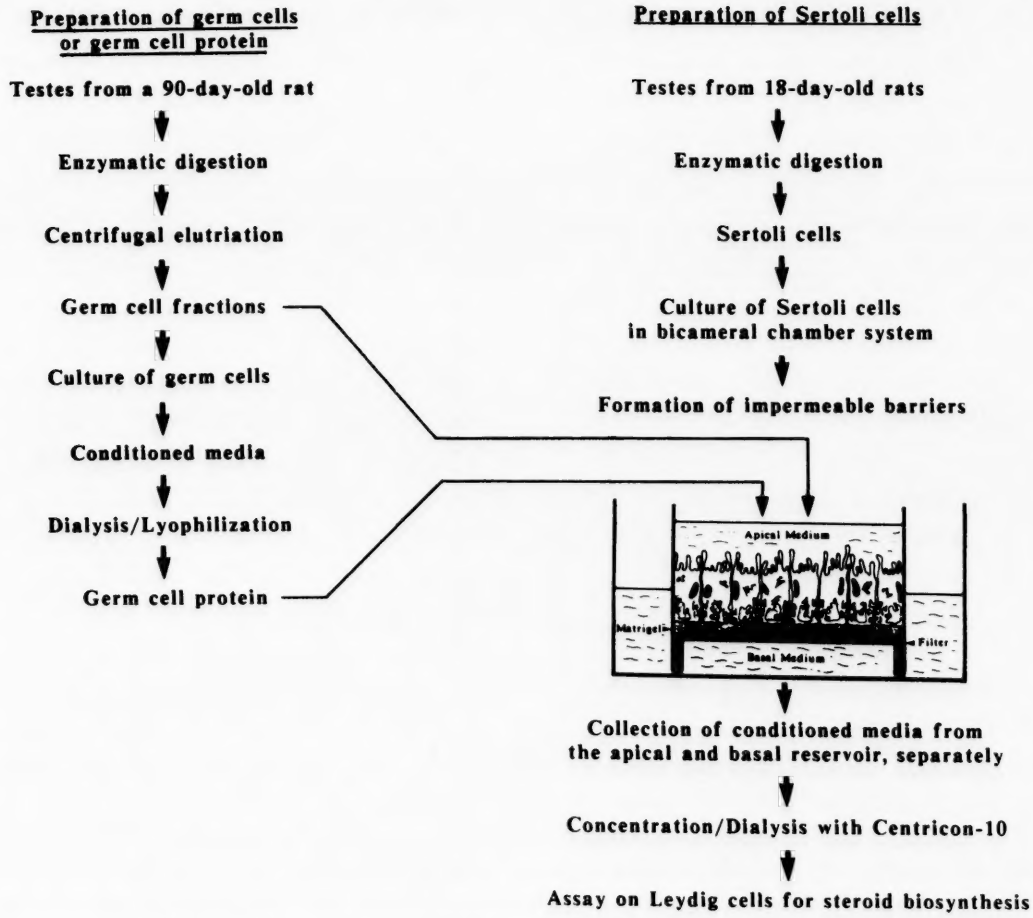

Fig. 1. Schematic representation of the experiments performed.

Leydig cell preparation and culture

Leydig cells from 70-day-old Sprague-Dawley rat testes were purified as previously described (Papadopoulos et al., 1986, 1987). The purity and the number of rat Leydig cells used in these experiments were histochemically determined by staining for 3β-hydroxysteroid dehydrogenase (3β-HSD) as previously described (Papadopoulos et al., 1985). MA-10 mouse tumor Leydig cells were maintained and used as previously reported (Ascoli, 1981; Papadopoulos et al., 1990). Rat Sertoli cell secreted proteins (rSCSP), prepared as described above, were tested for bioactivity on Leydig cells in a 2 h incubation period. Protein content of MA-10 Leydig cells, and the concentration of rSCSP, PSP and RSP were determined according to Bradford (1976) using γ-globulin as the standard. Testosterone and progesterone accumulation in the Leydig cell media (steroidogenic markers for rat Leydig and MA-10 cells, respectively) were measured by radioimmunoassay (Papadopoulos et al., 1987, 1990). Analysis of the radioimmunoassay (RIA) data was performed using the IBM-PC RIA Data Reduction program (version 4.1) obtained from Jaffe and associates (Silver Spring, MD, U.S.A.).

Statistics

Statistical analyses between control and treatment groups were performed by Student's t-test. Differences were considered significant when $p < 0.05$.

Results

Sertoli cell secretory protein stimulation of steroidogenesis

We and others have previously reported that immature rat Sertoli cells in conventional culture (on plastic dishes) secrete proteinaceous factor(s) which stimulate Leydig cell steroid biosynthesis (Janecki et al., 1985; Verhoeven and Cailleau, 1986; Ojeifo et al., 1990). The results presented in Fig. 2*A* confirm these observations. Moreover, in Fig. 2*B* it is shown that rSCSP stimulates MA-10 mouse tumor Leydig cell steroidogenesis with the same EC_{50} (25 μg/ml) as for the purified rat Leydig cells. Hence, this data validates the use of MA-10 Leydig cells for further studies on the regulation of the Sertoli cell secretory protein which stimulates Leydig cell steroidogenesis.

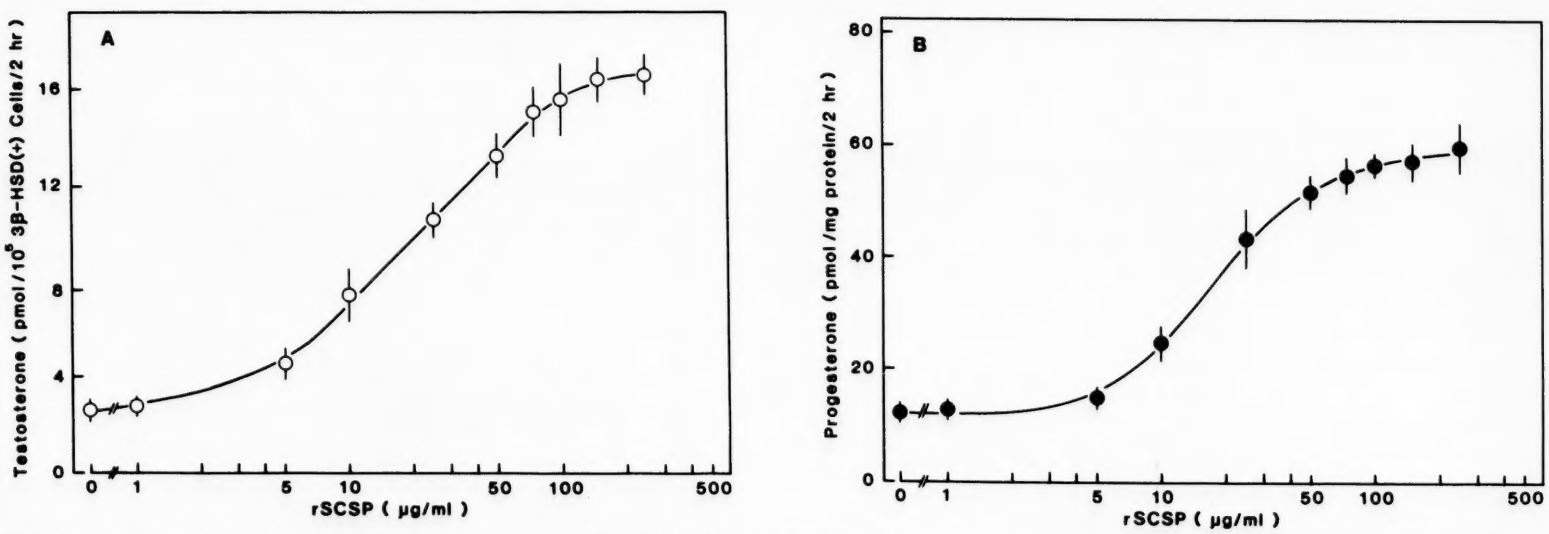

Fig. 2. Effect of rSCSP on purified normal rat Leydig cells (*A*) and MA-10 mouse tumor Leydig cells (*B*). rSCSP were collected from Sertoli cells cultured on plastic dishes as described under Materials and Methods. Leydig cells were incubated with the indicated concentrations of rSCSP for 2 h. At the end of the incubation, media were collected and analysed for steroid content (testosterone from rat Leydig cells and progesterone from MA-10 cells) by radioimmunoassay. The results obtained were normalized per unit of 3β-HSD positive cells and per unit cell protein for the rat and MA-10 Leydig cells, respectively. Values represent the mean $\pm$ SD obtained from two separate experiments ($n = 6$).

Polarized secretion of rSCSP which regulates Leydig cell steroidogenesis

Fig. 3*A* demonstrates that the Sertoli cell protein(s) which stimulates MA-10 Leydig cell steroid biosynthesis is secreted in a polarized manner from bicameral chambers. rSCSP collected from the apical and basal compartment of the chambers following 0–24 h incubation were tested for bioactivity on MA-10 Leydig cells by secretion of progesterone during a 2 h period. More than 80% of the rSCSP bioactivity which stimulated steroidogenesis was secreted in a basally polarized direction (Fig. 3*A*) in a time-dependent manner, reaching the maximum at 12 h.

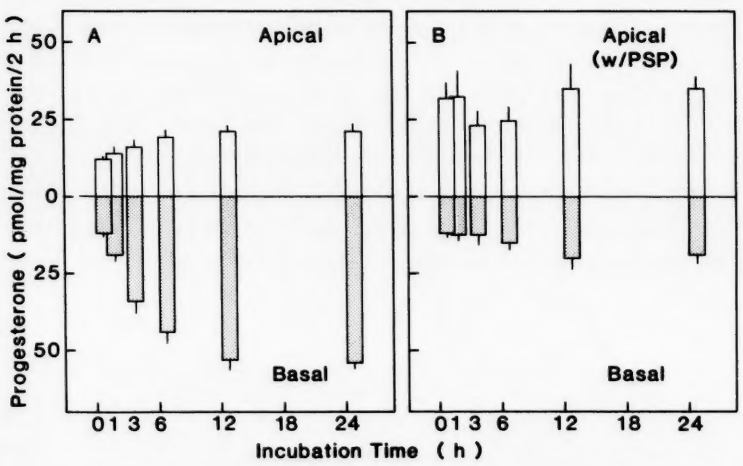

Fig. 3. Effect of PSP on the secretion by Sertoli cells of the protein(s) which stimulates Leydig cell steroid biosynthesis. Sertoli cells were cultured in the bicameral chamber system, as described under Materials and Methods. PSP (50 μg/ml) was added in the apical compartment of the chambers (*B*) and spent medium from both the apical and basal compartments was collected at the indicated time periods. The media, from each individual chamber, were then concentrated/dialysed against 10 mM sodium phosphate (pH 7.4) buffer and the basal and apical media tested for bioactivity on MA-10 Leydig cells cultured on 22 mm dishes. Progesterone accumulated in MA-10 cell media was measured by radioimmunoassay and the cell protein content was estimated as described under Materials and Methods. Open columns represent absolute amounts of progesterone produced by MA-10 cells in response to Sertoli cell proteins secreted into the apical chamber. Stippled columns represent absolute amounts of progesterone produced by MA-10 cells in response to Sertoli cell proteins secreted into the basal chamber. Values are means $\pm$ range from a representative experiment using two individual chambers. Qualitatively similar results were obtained in two other independent experiments.

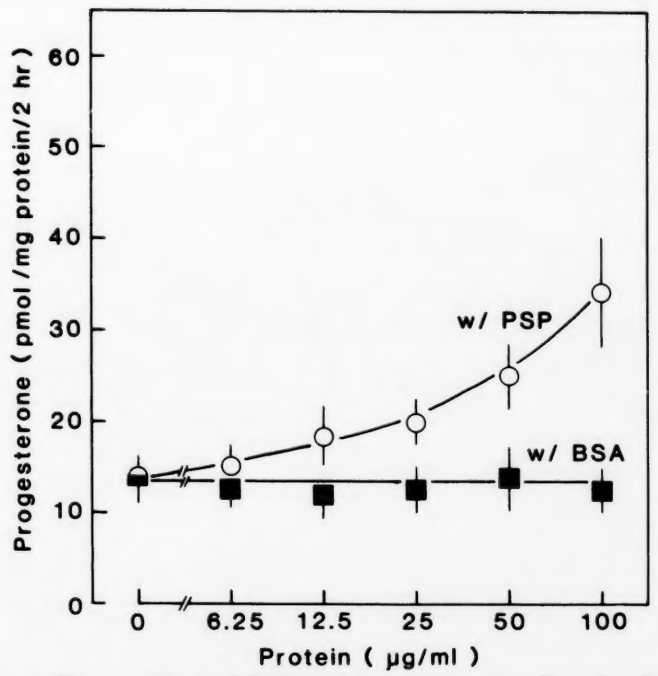

Fig. 4. Direct effect of PSP and bovine serum albumin (BSA) on Leydig cell steroidogenesis. MA-10 cells were incubated for 2 h with increasing concentrations of PSP and fatty-acid and globulin free BSA. Following incubation of MA-10 cells in PSP or BSA the media were collected and progesterone content estimated by radioimmunoassay. The results were normalized for MA-10 cell protein content. Data represent the mean $\pm$ SD obtained from two separate experiments ($n = 6$).

PSP modulation of rSCSP stimulatory effect on steroidogenesis

Following 24 h incubation of Sertoli cells in chambers with 50 μg/ml PSP, the viability of the Sertoli cells remained at virtually 100%, as indicated by transillumination of the Sertoli cells following trypan blue exclusion (data not shown). Following addition of 50 μg/ml PSP to the apical compartment of the chambers the rSCSP was collected from both the apical (with PSP) and basal chambers after 0–24 h incubation. This basally secreted rSCSP incubated with MA-10 cells for 2 h caused a 85% decrease ($p < 0.001$) of steroidogenesis in MA-10 cells (Fig. 3*B*) compared with basally secreted rSCSP prepared in the absence of PSP (Fig. 3*A*), whereas the apically secreted rSCSP (with PSP) caused a 2-fold increase ($p < 0.05$) in steroidogenesis of MA-10 cells (Fig. 3*B*). Increasing concentrations of PSP in the absence of rSCSP stimulated MA-10 Leydig cell progesterone production ($p < 0.001$) while bovine serum albumin (BSA), used as a control, was without any effect (Fig. 4). Hence, the stimulation observed using the

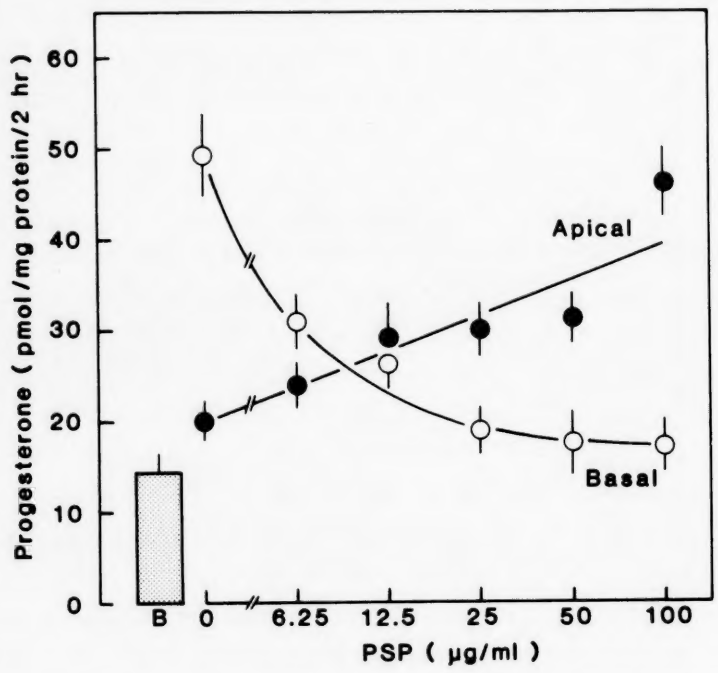

Fig. 5. Effect of increasing concentrations of PSP on the secretion of the Sertoli cell bioactive protein(s). PSP were placed on top of the Sertoli cell layer cultured in bicameral chambers for 24 h. Apical media (with PSP) and basal media containing rSCSP were collected, treated and tested for steroidogenic bioactivity as described in the legend to Fig. 3 and Materials and Methods. Column B (stippled) represents intrinsic progesterone production by MA-10 cells. Values are means ± range from a representative experiment using two individual chambers. Qualitatively similar results were obtained in two other independent experiments.

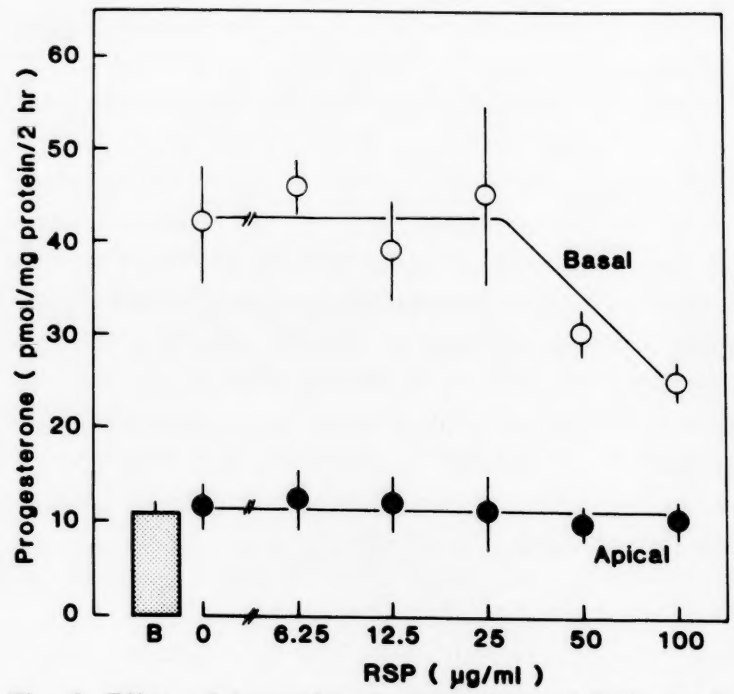

Fig. 7. Effect of increasing concentrations of RSP on the secretion of the Sertoli cell bioactive protein(s). RSP was placed on top of the Sertoli cell monolayer and cultured in the bicameral chambers for 24 h. Apical media (with RSP) and basal media containing the rSCSP were collected, treated and tested for steroidogenic bioactivity as described in the legend to Fig. 3. Column B (stippled) represents intrinsic progesterone production by MA-10 cells. Values are means ± SD from a representative experiment using three individual chambers. Qualitatively similar results were obtained in two other independent experiments.

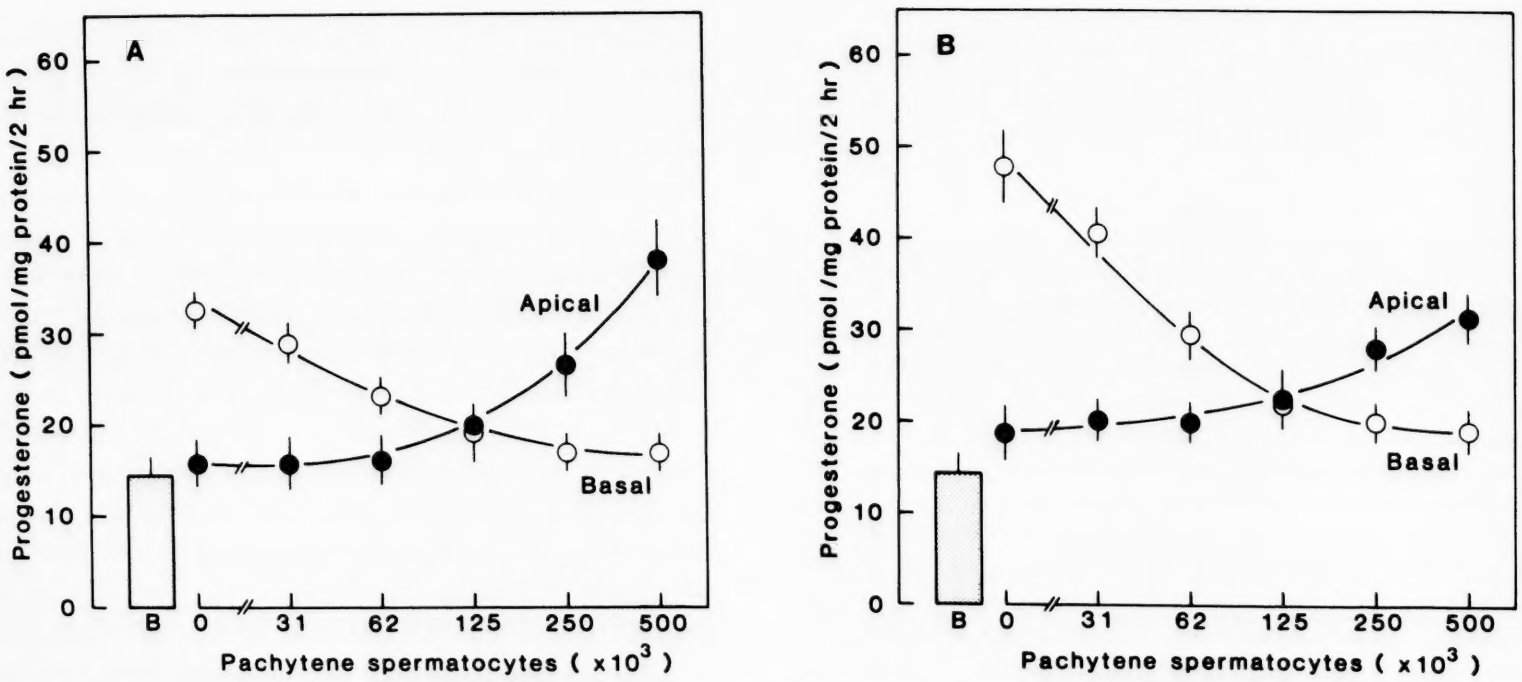

Fig. 6. Effect of PS on the secretion by Sertoli cells of the protein(s) which stimulates Leydig cell steroid biosynthesis. Increasing numbers of PS were added on top of the Sertoli cell layer and co-cultured in the bicameral chambers for 3 h (*A*) or 24 h (*B*). Apical and basal media were collected, treated and tested for bioactivity as described in the legend to Fig. 3 and Materials and Methods. Column B (stippled) represents the intrinsic progesterone production by MA-10 cells. Values are means ± range from a representative experiment using two individual chambers. Qualitatively similar results were obtained in two other independent experiments.

apical rSCSP, where the PSP were added (Fig. 3*B*), was due to the PSP as shown in Fig. 4. This indicates that PSP contain a protein(s) which has a steroidogenic effect on MA-10 Leydig cells. Moreover, increasing concentrations of PSP added to the apical compartment of the chambers, collected after 24 h and then tested for their effect on MA-10 Leydig cell steroidogenesis (Fig. 5), gave similar results as PSP added directly onto MA-10 Leydig cells (Fig. 4). These data clearly indicate that addition of PSP onto Sertoli cells does not affect the steroidogenic bioactivity of the apically secreted rSCSP. In Fig. 5 it is also shown that PSP decreased the basally secreted steroidogenic bioactivity in a concentration-dependent manner. At 50–100 μg/ml PSP, added on top of the Sertoli cells, the rSCSP collected from the basal compartment of the chambers had no effect on MA-10 Leydig cell steroidogenesis (Fig. 5).

In Fig. 6*A* and 6*B* the effect of rSCSP on MA-10 Leydig cell steroidogenesis following addition of freshly isolated PS to the apical compartment of the bicameral chambers over Sertoli cells and co-cultured for 3 h (Fig. 6*A*) or 24 h (Fig. 6*B*) incubation periods was examined. PS also inhibited in a time- and cell density-dependent manner the secretion of the Leydig cell stimulatory factor by Sertoli cells (Fig. 6*A* and *B*).

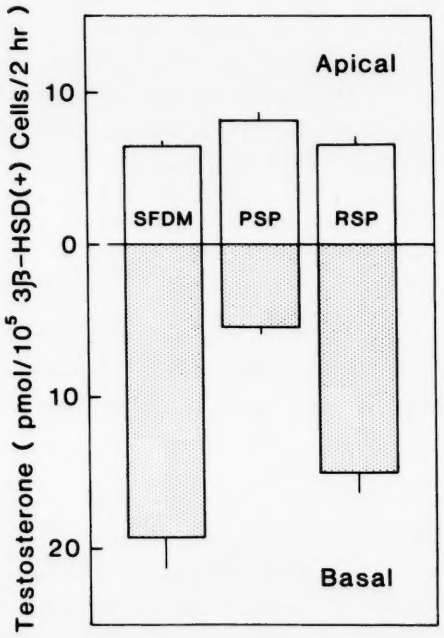

Fig. 8. Influence of the Sertoli cell steroidogenic modulatory protein secreted from either the apical (open columns) or basal (stippled columns) surface of Sertoli cells in bicameral chambers collected after incubation of the Sertoli cells for 24 h under control conditions (SFDM), or addition of 50 μg/ml PSP and 50 μg/ml RSP to the apical reservoir of the bicameral chambers. The Sertoli cell steroidogenic modulatory protein(s) was incubated with purified rat Leydig cells for 2 h and testosterone production normalized per 10^5 3β-HSD positive cells. Intrinsic Leydig cell testosterone production was 5.31 ± 0.10 pmol/10^5 cells. Values represent means ± SD from a representative experiment using three individual chambers. Qualitatively similar results were obtained from two other independent experiments.

In order to assess cell specificity, the effect of RSP on rSCSP modulation of MA-10 Leydig cell steroidogenesis was examined (Fig. 7). RSP had no effect on the apically and basally secreted Sertoli cell steroidogenic bioactivity, other than at the highest concentrations of RSP for the basally directed rSCSP steroidogenic bioactivity (Fig. 7). Similar results were obtained when purified Leydig cells (Fig. 8) were used to bioassay the rSCSP. As indicated in Fig. 8, the basally secreted rSCSP steroidogenic bioactivity (SFDM, Fig. 8) was not influenced by incubation of the Sertoli cells with RSP (RSP, Fig. 8), whereas addition of PSP to the apical chambers of the Sertoli cells grown in the bicameral chambers significantly ($p < 0.001$) inhibited the basally directed secretion of the rSCSP steroidogenic bioactivity (PSP, Fig. 8).

Discussion

The existence of proteinaceous factor(s) produced within the testis which modulate Leydig cell steroid biosynthesis has now been clearly established. It has been reported that rat interstitial fluid contains factor(s) which stimulate Leydig cell steroidogenesis (Sharpe and Cooper, 1984; Risbridger et al., 1986; Melsert et al., 1988; Jansz et al., 1990). Factor(s) with similar activity were shown to be secreted by seminiferous tubules in culture (Parvinen et al., 1984; Sharpe and Cooper, 1984; Papadopoulos et al., 1986, 1987) and by Sertoli cells in conventional culture on plastic dishes (Janecki et al., 1985; Verhoeven and Cailleau, 1986, 1990; Papadopoulos et al., 1987; Ojeifo et al., 1990). The physiological relevance of such factor(s) is as yet uncertain, although they may be potentially important in relation to maintenance of the high levels of intratesticular testosterone required for prepubertal development of the seminiferous epithelium and for the quantitative development and maintenance of normal spermatogen-

esis in the adult. A cyclic variation in the secretion of Sertoli cell proteins, dependent on the types of germ cells in contact with the Sertoli cells has been elegantly demonstrated by several laboratories (Lacroix et al., 1981; Parvinen, 1982; Wright et al., 1983; Shabanowitz et al., 1986). Recent studies indicate that pachytene spermatocytes and other germ cell types have a direct effect on Sertoli cell function (Galdieri et al., 1984; Le Magueresse et al., 1986, 1988; Djakiew and Dym, 1988; Janecki et al., 1988; Castellon et al., 1989; Onoda and Djakiew, 1990, 1991). Cyclic variations in the secretion of Sertoli cells protein(s) which modulate Leydig cell steroidogenesis have also been demonstrated (Parvinen et al., 1984; Syed et al., 1985; Kamtchouing et al., 1988; Vihko and Huhtaniemi, 1989). Several laboratories have shown that disruption of spermatogenesis by germ cell depletion causes an increase in the steroidogenic bioactivity of the interstitial fluid when compared with the interstitial fluid from intact testes (Sharpe and Cooper, 1984; Bartlett and Sharpe, 1987; Risbridger et al., 1987; Jansz et al., 1990). Moreover, seminiferous tubule conditioned medium collected from germ cell-depleted tubules contains more steroidogenic bioactivity compared to medium derived from control tubules in culture (Papadopoulos et al., 1987). Hence, it appears that germ cells may inhibit the secretion of the biologically active protein(s) from Sertoli cells (Papadopoulos et al., 1987). However, direct evidence of this interaction between germ cells, Sertoli cells and Leydig cells has been lacking. We report here that polarized Sertoli cells cultured in vitro on a permeable support (filter) secrete in a basal direction the factor(s) which stimulates Leydig cell steroidogenesis and we provide direct evidence that PS inhibit the secretion of this factor(s) from Sertoli cells. Addition of PSP and PS to the apical compartment of the chamber inhibited in a time- and concentration-dependent manner the secretion by Sertoli cells of the protein(s) which stimulate Leydig cell steroidogenesis. The observation that PSP alone stimulated in a concentration-dependent manner, at maximum 2-fold, Leydig cell steroid production indicates that PSP may contain an activator protein, the identity and physiological significance of which remain to be investigated.

Sertoli cells in vivo are tall columnar cells with a highly polarized cytoplasm in contact, at their basal surface, with a basement membrane matrix (Dym, 1977). Conditions for the culture and maintenance in vitro of Sertoli cells morphologically similar to those in vivo have been recently developed using the bicameral culture chamber system (Byers et al., 1986; Janecki and Steinberger, 1986). Using this culture system different investigators reported the polarized secretion of different Sertoli cell products (Hadley et al., 1987; Janecki and Steinberger, 1987; Handelsman et al., 1989). However, differences in the results obtained between some laboratories, concerning the polarized secretion of different Sertoli cell products, prompted us to further characterize the conditions for Sertoli cell culture in the bicameral chambers (Onoda et al., 1990) which are necessary for the formation of an impermeable confluent monolayer. Under these conditions the cells of the Sertoli cell epithelial sheet are able to prevent hydrodynamic equilibration of fluids between the apical and basal reservoir of the bicameral chambers thereby preventing the mixing of fluids between the chambers and hence maintaining a functional compartmentalization of the polarized secretory products. Indeed, prevention of the mixing of fluids between the apical and basal compartments of the bicameral chambers is particularly important for these experiments in relation to collection of polarized Sertoli cell secretory proteins (apical vs. basal, Fig. 3*A* and *B*), and the direct action of PSP on stimulating steroidogenesis (Fig. 4), compared to the action of PSP on Sertoli cell mediated modulation of Leydig cell steroid biosynthesis (Figs. 5 and 6). In this respect, only those Sertoli cell cultures which maintained an hydrodynamic permeability barrier (Onoda et al., 1990) were used for these experiments. It is important to note that addition of PSP to the apical compartment did not affect the Sertoli cell hydrodynamic permeability barrier. This is shown by the absence of any stimulatory activity of the basal rSCSP after addition of 100 μg/ml PSP apically, the later of which stimulated Leydig cell steroid production approximately 2-fold (Fig. 5).

The MA-10 Leydig cell line was used in the most part as a bioassay system in the present studies. Both purified rat and MA-10 Leydig cells, respectively, responded similarly in relation to

steroid production, similar EC_{50} and to increasing concentrations of rSCSP all of which indicate that rSCSP activates the same pathway in both models. The major difference between rat and MA-10 Leydig cells is that the MA-10 cells lack the enzyme 17-hydroxylase for metabolism of progesterone to testosterone. Nevertheless, both cell types respond to LH by producing increased amounts of steroids (Ascoli et al., 1981; Papadopoulos et al., 1985, 1987, 1990). Moreover, in preliminary studies using partially purified preparations of human and rat Sertoli cell secreted proteins on human, rat, and MA-10 Leydig cells, it was observed that SCSP has no action on cAMP production, but stimulated the mobilization and/or conversion of cholesterol to pregnenolone, the precursor of all steroids (unpublished results). Hence, all these observations provide validity for use of the MA-10 Leydig cell line as a bioassay system for our studies. Moreover, the results obtained using MA-10 Leydig cells were similar to results obtained with the purified rat Leydig cells suggesting that these observations are applicable to the physiological regulation of steroidogenesis.

The present data demonstrate that PS, present in all stages of the seminiferous epithelium, inhibit the secretion by Sertoli cells of the Leydig cell stimulatory protein(s). The inhibition of steroidogenic bioactivity in basally directed rSCSP by the highest concentrations of RSP could be accounted for by concentration of PSP from PS contaminants in the RS fraction, or by a much smaller amount of this protein in RSP relative to PSP. In any event, there is approximately an order of magnitude difference in PSP and RSP with respect to modulation of the basally directed rSCSP steroidogenic bioactivity, indicating that germ cell specificity for regulation of the rSCSP modulator of Leydig cell steroidogenesis resides predominantly in the PS. The observation that the Sertoli cell protein(s) stimulating Leydig cell steroidogenesis is secreted in a basal direction suggest that in vivo this protein(s) will occur in the interstitial fluid which permeates the interstitial spaces of the testis and which separates the seminiferous tubules from the Leydig cells (Fawcett et al., 1973; Clark, 1976). Our observations further support the hypothesis that the Sertoli cell secreted stimulatory protein(s), the seminiferous tubule bioactive factor(s), and the interstitial fluid bioactivity share the same protein(s), the secretion of which is regulated by pachytene spermatocytes, whereupon it is secreted from the basal compartment of the tubules and diffuses in the interstitial fluid where this protein(s) interacts with the Leydig cells.

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Abnormal metabolism of polyunsaturated fatty acids in adrenal glands of diabetic rats

Ruben A. Igal, Elisabet C. Mandon * and Irma N.T.de Gómez Dumm *

Instituto de Investigaciones Bioquímicas de La Plata (INIBIOLP), CONICET-UNLP, 1900 La Plata, Argentina

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Summary

Studies carried out on the adrenal glands of experimental diabetic rats have shown an important inhibition in polyenoic fatty acid biosynthesis. This effect was demonstrated by testing the activities of long-chain fatty acyl-CoA synthetase, the Δ^5- and Δ^6-desaturases of the (n–6) essential fatty-acid series and the Δ^6-desaturase of the (n–3) series in liver and adrenal microsomes. The depression in desaturating activity in the insulin-deprived animals was independent of that produced on acyl-CoA-thioester biosynthesis. Experiments measuring the incorporation and transformation of [1-^{14}C]eicosa-8,11,14-trienoic acid in adrenocortical cells isolated from streptozotocin-diabetic animals demonstrated a significant inhibition of arachidonic acid biosynthesis compared to controls. Insulin injections in diabetic rats partially restored the Δ^5- and Δ^6-desaturase activities. This effect could result from direct action by the hormone since the restoration was reproduced when arachidonic acid biosynthesis was measured after insulin was added to the incubation medium of adrenocortical cells isolated from diabetic animals. The results of the present study provide new information about the implication of this abnormal metabolism in the adrenal gland of diabetic rats.

Introduction

It has long been recognized that insulin deficiency in animals is associated with impaired lipogenesis within liver and adipose tissue. With respect to polyunsaturated fatty acids, there is a depression in the biosynthesis of the aforementioned fatty acids in experimental diabetic rats. This failure primarily occurs at the level of the desaturases, the principal enzymes in this pathway under hormonal regulation (Eck et al., 1979; Prasad and Joshi, 1979). Liver microsomes from diabetic animals have a reduced ability to desaturate stearic acid to oleic acid (Δ^9-desaturase) (Gellhorn and Benjamin, 1964), linoleic acid to γ-linolenic acid (Δ^6-desaturase) (Mercuri et al., 1966; Faas and Carter, 1980), α-linolenic acid to octadecatetraenoic acid (Δ^6-desaturase) (Mercuri et al., 1967) and eicosa-8,11-dienoic acid to eicosa-5,8,11-trienoic acid (Δ^5-desaturase) (Castuma et al., 1972).

Address for correspondence: Ruben A. Igal, Instituto de Investigaciones Bioquímicas de La Plata (INIBIOLP), CONICET-UNLP, Calles 60 y 120, 1900 La Plata, Argentina.

* Members of the Consejo Nacional de Investigaciones Científicas y Técnicas, Argentina.

The study of polyunsaturated fatty acid biosynthesis and its correlation with cellular physiology in diabetes has thus far been focused primarily on liver. However, indirect evidence for an abnormal fatty acid biosynthesis was obtained in heart, skin, testes, kidney, adrenal gland and pancreas in experimental animals (Peluffo et al., 1970; Holman et al., 1983; Huang et al., 1984; Kanzaki et al., 1987; Levy et al., 1988) and in insulin-dependent diabetic humans (Miklailidis et al., 1986). In the example of the adrenal gland, there also occurs an abnormal regulation of the factors that control adrenal steroid secretion and metabolism in streptozotocin (STZ)-diabetic rats (De Nicola et al., 1976, 1977). Acute diabetes was accompanied by adrenal hypertrophy and high plasma corticosterone levels (De Nicola et al., 1976). This effect could have been produced by steady stimulation of the hypothalamic–pituitary–adrenal axis with a continuous release of adrenocorticotrophin (ACTH) (L'Age et al., 1974).

A characteristic of adrenal cortical cells 'in vivo' is their acute steroidogenic response to ACTH with a notable depletion in the lipid droplets that are rich in cholesterol esters (Garren et al., 1971; Boyd and Trzeciak, 1973). In this regard, other interesting observations in this type of cell is that the cholesterol ester fraction contains large amounts of polyunsaturated fatty acids, mainly 20 : 4(n–6) and 22 : 4(n–6) (Vahouny et al., 1978). Moreover, in rats, the administration of ACTH 'in vivo' or the addition of the hormone to the incubation medium of adrenocortical cells, produces a significant decrease in the activity of the fatty acid desaturases (Mandon et al., 1987).

In view of the aforementioned data, the present study deals with the effect of STZ-induced diabetes on the biosynthesis of polyunsaturated fatty acids and also on the fatty acid profile of the adrenal glands. Finally, the effect of insulin replacement on these biochemical changes was also tested.

Materials and methods

Chemicals

[1-^{14}C]Linoleic acid (59 mCi/mmol), [1-^{14}C]α-linolenic acid (56 mCi/mmol) and [1-^{14}C]eicosa-8,11,14-trienoic acid (47.7 mCi/mmol) were purchased from Amersham International, U.K. All the acids were 98–99% radiochemically pure. Unlabelled acids were supplied by Nu-Chek Prep., Elysian, MN, U.S.A. NADH, ATP, CoA, collagenase type IV and deoxyribonuclease I were provided by Sigma Chemical Co., St. Louis, MO, U.S.A. Streptozotocin (STZ) was kindly donated by Upjohn Laboratories, Kalamazoo, MI, U.S.A. The bovine NPH insulin (Betasint) used was from Lab. Beta, Buenos Aires, Argentina. All other chemicals were of analytical grade.

Treatment of animals

The female Wistar rats used, weighing 150–200 g, were maintained on a commercial diet (Cargill type C) and water ad libitum. Diabetes was induced in non-fasted rats, by the intravenous injection of 70 mg/kg of STZ freshly dissolved in citrate buffer (pH 4.5). Control rats received an equivalent amount of citrate saline. A week later, STZ-treated rats were fasted for 12 h and blood was collected from the orbital venous sinus for glucose determination by a commercial enzymatic method (Wiener Lab. Test, Rosario, Argentina). Only those rats with blood glucose greater than 300 mg/dl were considered diabetic (Mercuri et al., 1966).

As diabetic animals usually gain less weight than controls, despite having hyperphagia, the food intake of the control rats was restricted in order to offset this discrepancy; with the end results that the body weights of both groups of animals were comparable throughout the course of the studies.

Insulin was injected subcutaneously (4 U/rat) at 8 a.m. and 8 p.m. daily for 2 days prior to death (Eck et al., 1979). In order to avoid individual differences among the animals, one day before they were sacrificed, the rats of all groups were fasted for 12 h, refed for 2 h, and then fasted for other 12 h.

Isolation of microsomes and enzyme assays

Rats were divided into three experimental groups of 21 animals each (control, diabetic and diabetic treated with insulin). The animals were killed by decapitation 10 days after STZ or saline injection. Blood was drained off and collected for glucose, triglycerides (Nakazawa and Mead, 1976), total cholesterol (Huang et al., 1961) and corti-

costerone (Sayers et al., 1971) determinations and also to measure plasma fatty acid composition. Adrenal glands of all the rats within each group were pooled to obtain the microsomal fraction. To separate liver microsomes, three pools of seven rats each per group were prepared. Livers and adrenal glands (free of fat and decapsulated) were rapidly excised, weighed and placed in ice-cold homogenizing solution (1 : 3, w/v) containing 0.25 M sucrose, 62 mM phosphate buffer, pH 7.0, 0.15 M KCl, 5 mM $MgCl_2$ and 100 μM EDTA. The microsomal fraction was separated by differential centrifugation at $105{,}000 \times g$ as described previously (Castuma et al., 1972). Protein content was measured by the method of Lowry et al. (1951).

The desaturation of the fatty acids of liver and adrenal microsomes was measured by estimation of the percentage conversion of [1-^{14}C]linoleic acid to γ-linolenic acid, [1-^{14}C]α-linolenic acid to octadeca-6,9,12,15-tetraenoic acid and [1-^{14}C]eicosa-8,11,14-trienoic acid to arachidonic acid. 4 nmol of the labelled acid and 96 nmol of the corresponding unlabelled acid were incubated with 5 mg of liver microsomal protein. Adrenal microsomal Δ^6 desaturation was measured by incubating 1.5 mg of protein and 20 nmol (13.4 μM) of labelled linoleic or α-linolenic acids. The Δ^5 desaturation activity was measured with 1 mg of adrenal microsomal protein and 15 nmol (10.0 μM) of labelled eicosa-8,11,14-trienoic acid by the procedure described in a previous work (Mandon et al., 1987). Under these experimental conditions, the desaturation activity was linearly related to protein concentration and the enzymes were completely saturated with the corresponding substrates (Mandon et al., 1986). The incubation medium contained 4 μmol of ATP, 0.1 μmol of CoA, 1.25 μmol of NADH, 5 μmol of $MgCl_2$, 2.42 μmol of *n*-acetyl cysteine, 62.5 μmol of NaF, 0.5 μmol of nicotinamide and 62.5 μmol of phosphate buffer (pH 7) in a total volume of 1.5 ml of 0.15 M KCl, 0.25 M sucrose solution, per tube. Details of the incubation procedure, lipid extraction and the method to obtain the fatty acid methyl esters and their identification were described previously (Mandon et al., 1987).

The assay used to measure the long chain fatty acyl-CoA synthetase activity from liver and adrenal microsomes was similar to that described by Tanaka et al. (1979), which procedure briefly consisted in the enzymatic conversion of heptane-extractable radiolabelled fatty acid into water-soluble acyl-CoA esters, measured in triplicate. Acyl-CoA synthetase activity measurement with various long chain fatty acid substrates and its kinetic properties in adrenal microsomes were detailed in a previous report (Mandon et al., 1988).

Isolation of adrenocortical cells and measurement of arachidonic acid biosynthesis

To obtain adrenocortical cells, diabetic and control rats were separated into groups of ten animals each. The adrenal glands of each group were removed immediately after decapitation, pooled and kept in Krebs-Ringer bicarbonate buffer (KRB), pH 7.4, supplemented with 0.2% glucose and 0.5% albumin. Livers of the same animals were also removed to measure microsomal fatty acid desaturation activity simultaneously. The adrenals were decapsulated and the isolation of the cells from the zona fasciculata-reticularis was carried out by mechanical agitation in siliconized glassware with collagenase and deoxyribonuclease, according to the procedure of Ray and Strott (1978). The pellets of the adrenocortical cells, resuspended in KRB glucose (0.2%), were counted in a hemocytometer. Viability of dissociated cells was assessed by exclusion of trypan blue (Jauregui et al., 1981) and it was routinely 85–90%.

Aliquots of the isolated cell suspensions (80,000 cells per tube) were incubated in a final volume of 1.5 ml of KRB glucose in siliconized glassware in a metabolic shaker (80 oscillations/min) at 37 °C under an atmosphere of 95% O_2/5% CO_2 for 30, 90 and 180 min. The sodium salt of [1-^{14}C]eicosa-8,11,14-trienoic acid (6.7 μM concentration) was added to defatted albumin according to Spector et al. (1965). In those experiments where the effect of insulin was tested, the hormone was added to the incubation medium (1 mU) (Swallow and Sayers, 1969; Kitabchi and Sharma, 1971) simultaneously with the labelled 20 : 3(n–6) acid. At the end of the incubation, the cell suspensions were transferred to tubes at 4 °C and rapidly centrifuged. Aliquots of the incubation medium of adrenocortical cells were separated to measure corticosterone levels. The cell pellet was washed twice with cold 0.85% (w/v) NaCl solution and then

resuspended in the same solution. An aliquot of each suspension was used to measure the amount of cellular protein (Lowry et al., 1951). The remnant was centrifuged at $2000 \times g$ for 5 min. The supernatant solution was discarded and the cells were saponified and esterified to recover the methyl esters as shown previously (Mandon et al., 1986). The radioactivity of the recovered methyl esters was determined in a Beckman liquid scintillation counter (model L5-3133 P) with 96.7% efficiency for ^{14}C. The distribution of radioactivity among the fatty acids was determined in a gas-liquid radiochromatograph equipped with a Panax proportional counter and a Suroscribe 2S recorder. The column was packed with 10% SP-2330 coated on Chromosorb WAW-DMCS (100–200 mesh).

Total fatty acid analysis

Lipids were extracted from aliquots of plasma, liver and adrenal microsomes with chloroform/methanol (2:1, v/v) following the procedure of Folch et al. (1957). The fatty acids were converted to methyl esters and analyzed in a Hewlett-Packard model 5840 A gas liquid chromatograph equipped with a flame ionization detector following the procedure described in a previous report (Mandon et al., 1986).

Statistical analysis

The results were tested for statistical significance by the Student *t*-test compared to the respective control.

Results

Plasma biochemical parameters in control, diabetic and insulin-treated diabetic rats

At the time of sacrifice, diabetic rats had significantly higher levels of plasma glucose as well as triglycerides, total cholesterol and corticosterone than control and insulin-treated diabetic rats (Table 1).

TABLE 1

BIOCHEMICAL PLASMA PARAMETERS IN CONTROL, DIABETIC AND INSULIN-TREATED DIABETIC RATS

Each value represents the mean of 21 determinations ± 1 SEM.

Rat group	Glucose (mg/dl)	Triglycerides (mg/dl)	Total cholesterol (mg/dl)	Corticosterone (μg/dl)
Control	113 ± 15	70 ± 6	89 ± 7	10 ± 3
Diabetic	550 ± 60 [b]	210 ± 11 [b]	125 ± 6 [a]	36 ± 8 [a]
Insulin-treated diabetic	84 ± 10 [d]	51 ± 3 [d]	60 ± 10 [c]	20 ± 3 [c]

Significant differences between the control and diabetic rats: [a] $P < 0.01$, [b] $P < 0.001$. Significant differences between the diabetic and insulin-treated diabetic rats: [c] $P < 0.01$, [d] $P < 0.001$.

Desaturation activity in liver and adrenal microsomes of diabetic and insulin-treated diabetic rats

Fig. 1 (*A* and *B*) illustrates the activity of Δ^6-desaturase in both liver and adrenals, as measured by the transformation of α-linolenic acid to octadeca-6,9,12,15-tetraenoic acid and linoleic acid to γ-linolenic acid. In either tissue, Δ^6 desaturating activity decreased in the microsomes of diabetic rats by approximately 60–70% compared to the respective controls. In diabetic rats subjected to insulin injections, there was a compensatory increase in the desaturation of linoleic and α-linolenic acids that partially restored the enzymatic activity to control values. This recovery of Δ^6 desaturating activity was higher in the liver than in the adrenal gland.

The conversion of eicosatrienoic acid to arachidonic acid is shown in Fig. 1*C*. Streptozotocin-treated rats showed a significant decrease in microsomal Δ^5 desaturating activity of both tissues, although this depression was greater with respect to liver. Insulin administration to diabetic rats produced a significant, albeit incomplete, restoration of Δ^5 desaturating activity in both tissues.

Long chain acyl-CoA synthetase activity in adrenal and liver microsomes of control, diabetic and insulin-treated diabetic rats

Fig. 2 shows that the thioesterification of labelled polyunsaturated fatty acid was significantly depressed in the microsomes of liver and adrenal gland of diabetic animals. The extent of the depression of acyl-CoA synthetase activity was similar in both tissues except for the synthesis of the linolenyl CoA thioester in adrenal micro-

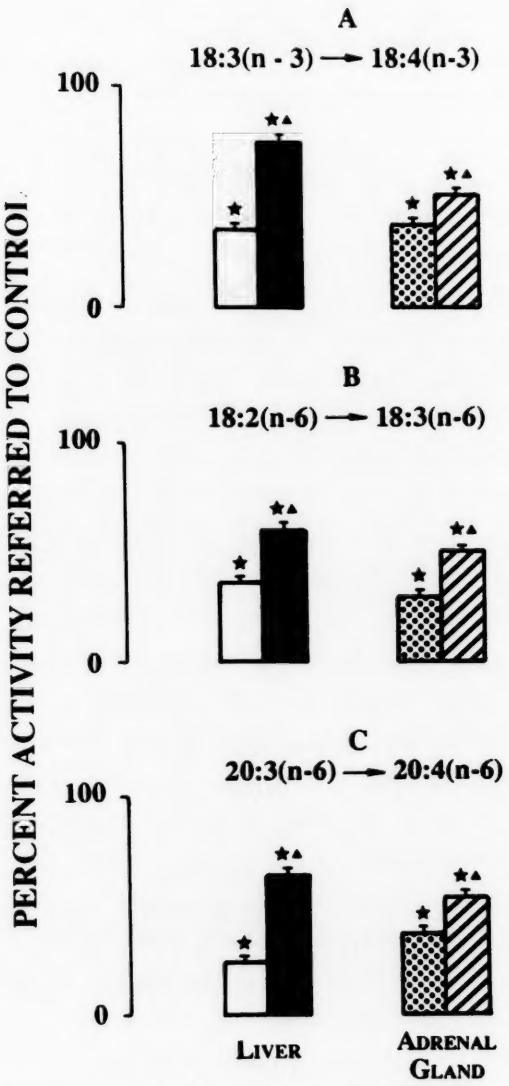

Fig. 1. Conversion of labelled α-linolenic acid to octadeca-6,9,12,15-tetraenoic acid (*A*), linoleic acid to γ-linolenic acid (*B*) and eicosa-8,11,14-trienoic acid to arachidonic acid (*C*) in microsomes of liver (open columns) and adrenal gland (stippled columns) from diabetic rats, and of liver (filled columns) and adrenal gland (hatched columns) from insulin-treated diabetic rats. Control enzymatic activities (nmol/mg protein/min) were: (*A*) liver 0.663 ± 0.043, adrenal gland 0.718 ± 0.031; (*B*) liver 0.772 ± 0.050, adrenal gland 0.575 ± 0.030; and (*C*) liver 0.790 ± 0.050, adrenal gland 0.590 ± 0.040. Results are the mean of three experiments. Vertical lines represent ± 1 SEM. Symbols above columns indicate statistically significant differences from values of control rats (asterisks) and from values of diabetic untreated rats (triangles) at $P < 0.001$.

somes, where the inhibition was more evident. Insulin treatment of diabetic rats, however, produced no modification in the decrease in long chain acyl-CoA synthetase activity seen in the diabetic state.

Total fatty acid composition of plasma, liver and adrenal gland in diabetic rats and in those diabetic animals treated with insulin

Because the changes observed in plasma and liver fatty acid composition in diabetic and insulin-treated diabetic rats were similar to those already published (Mercuri et al., 1967; Faas and Carter, 1980, 1983; Holman et al., 1983; Kanzaki et al., 1987; Levy et al., 1988), these data are not shown in this paper. The results, however, may be

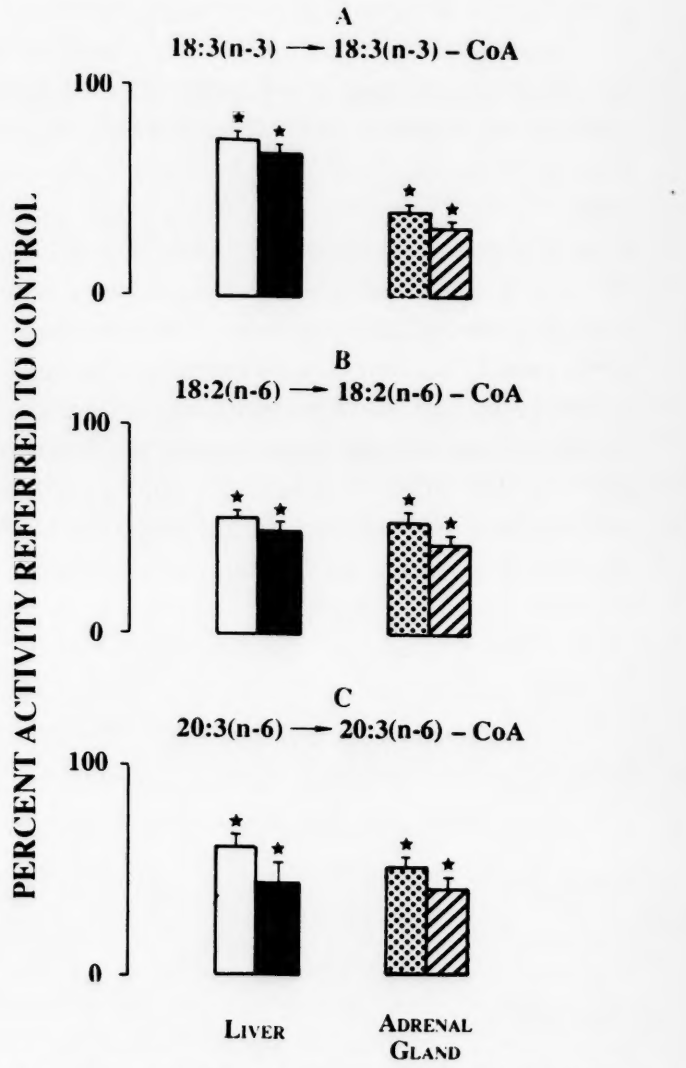

Fig. 2. Long chain acyl-CoA synthetase activity for different labelled substrates: α-linolenic acid (*A*), linoleic acid (*B*) and eicosa-8,11,14-trienoic acid (*C*) in microsomes of liver (open columns) and adrenal gland (stippled columns) from diabetic rats, and of liver (filled columns) and adrenal gland (hatched columns) from insulin treated rats. Control enzymatic activities were (nmol/mg protein/min): (*A*) liver 25.4 ± 0.2, adrenal gland 36.5 ± 2.0; (*B*) liver 34.2 ± 0.3, adrenal gland 49.0 ± 0.3; and (*C*) liver 17.5 ± 0.2, adrenal gland 38.4 ± 0.6. Results are the mean of three experiments. Vertical lines represent ± 1 SEM. Asterisk above columns indicates significant differences from control values at $P < 0.001$.

summarized as follows: (1) The most important changes observed in the plasma of diabetic rats as compared to control animals were an increase in the proportion of linoleic acid and a decrease in that of arachidonic acid. These deviations were corrected by insulin replacement. (2) The liver fatty acid composition of the diabetic rats showed an increase in 18 : 0 and 18 : 2(n–6) acids concomitant with a decrease in 18 : 1(n–9) and 20 : 4(n–6) acids, compared to the non-diabetic controls. No significant changes were detected in other fatty acids. (3) Treatment of rats with insulin led to a recovery of the normal fatty acid distribution.

The percentage composition of the fatty acids in the adrenal gland of control rats showed a pattern consistent with that previously reported (Mandon et al., 1986). The major fatty acids are represented by stearic and arachidonic acids along with a significant amount of 22 : 4(n–6) acid (Table 2). In diabetic animals there were significant increases in the percentages of stearic and linoleic acids and a marked decrease in oleic and arachidonic acids. No significant modifications in the other fatty acids were observed. Insulin treatment completely reversed the alterations seen in the diabetic group, returning the percentages of the fatty acids to values statistically indistinguishable from those obtained in control rats. 18 : 1/18 : 0 and 20 : 4(n–6)/18 : 2(n–6) ratios showed a near 50% depression in diabetic adrenal microsomes, which effect was totally prevented by insulin supplementation.

Incorporation and desaturation of [1-^{14}C]eicosa-8,11,14-trienoic acid in isolated adrenocortical cells of control and diabetic rats

In order to investigate the nature of the abnormality in fatty acid metabolism in diabetic rats, adrenocortical cells were isolated and the incorporation and transformation of eicosatrienoic acid measured (Fig. 3). The left half of the figure indicates the incorporation of 20 : 3(n–6) acid (upper panel) and the desaturation of 20 : 3(n–6) to 20 : 4(n–6) acids (lower panel) by cells from control and diabetic animals. The right half of the figure was taken from the respective data in the left half and represents the variations in these parameters after reexpression of the data as a percent of the control values. The incorporation of 20 : 3(n–6) acid in the adrenocortical cells was slightly higher (20%) in the control rats than in the diabetic animals after only 30 min of incubation. This incorporation increased with time in both groups, but it did no more markedly in cells from control rats than in those from diabetic animals after 90 and 180 min of incubation. Thus, at these later time points, the inhibition of 20 : 3(n–6) incorporation increased consistently to a level of

TABLE 2

FATTY ACID COMPOSITION OF TOTAL LIPIDS IN RAT ADRENAL MICROSOMES

Results are the mean of three pools of adrenal glands obtained from a total of 21 rats ± SEM expressed as μg% of total fatty acids.

Fatty acid	Control (μg%)	Diabetic (μg%)	Insulin-treated diabetic (μg%)
16 : 0	6.2 ± 0.2	5.7 ± 0.1	5.9 ± 0.1
18 : 0	33.8 ± 0.1	40.8 ± 0.1 [a]	34.8 ± 0.4 [d]
18 : 1	7.1 ± 0.3	4.8 ± 0.1 [b]	8.0 ± 0.2 [d]
18 : 2(n–6)	5.3 ± 0.1	7.6 ± 0.3 [b]	4.6 ± 0.5 [c]
20 : 4(n–6)	40.0 ± 0.3	34.0 ± 0.3 [a]	39.5 ± 0.1 [d]
22 : 4(n–6)	4.8 ± 0.1	4.2 ± 0.3	4.6 ± 0.1
22 : 5(n–6)	0.8 ± 0.1	0.5 ± 0.1	0.6 ± 0.2
22 : 5(n–3)	1.6 ± 0.2	2.2 ± 0.2	1.7 ± 0.3
22 : 6(n–3)	0.5 ± 0.1	0.3 ± 0.1	0.3 ± 0.1
18 : 1/18 : 0	0.21	0.12	0.23
20 : 4/18 : 2	7.55	4.47	8.59

Significant difference between the control and diabetic rats: [a] $P < 0.01$, [b] $P < 0.001$.
Significant difference between the diabetic and insulin-treated diabetic rats: [c] $P < 0.01$, [d] $P < 0.001$.

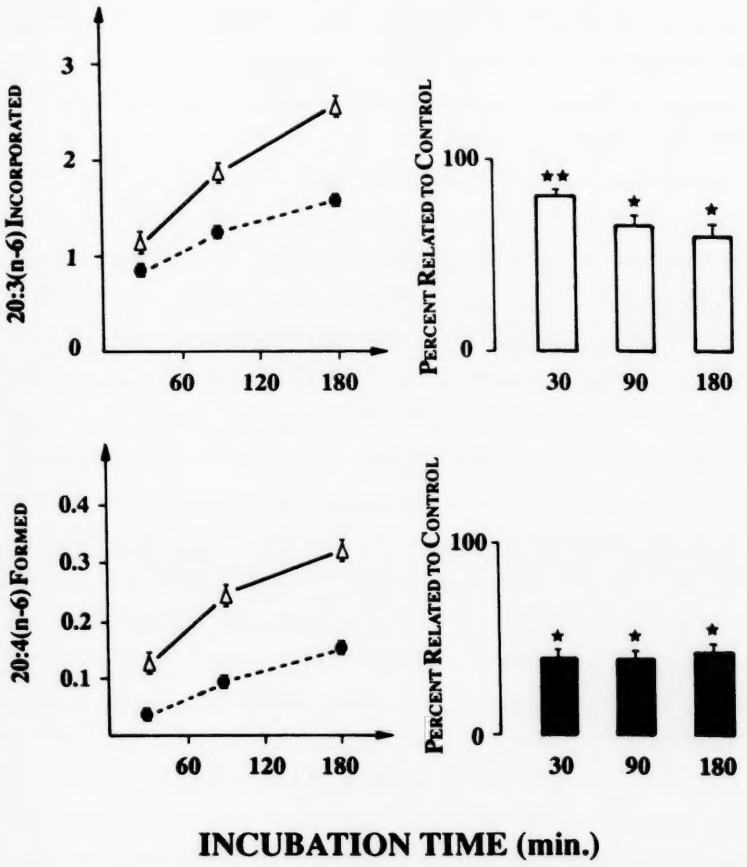

Fig. 3. Effect of diabetes on the incorporation (upper panel) and transformation (lower panel) of [1-^{14}C]eicosa-8,11,14-trienoic acid in isolated adrenocortical cells, as a function of incubation time. Left half of the figure: cells isolated from control (open triangles) and from diabetic (closed circles) rats. Values are expressed as nmol/mg protein and are the mean of three determinations $\pm$ 1 SEM. Right half of the figure: respective data for the diabetic animals from the left half replotted as a percent of control values. Open columns represent the incorporation of 20 : 3(n–6) acid and filled columns the transformation of 20 : 3(n–6) to 20 : 4(n–6) acid. Vertical lines represent $\pm$1 SEM. Asterisks indicate significant differences from control values: ** $P < 0.01$, * $P < 0.001$. The incubation conditions are described in Materials and Methods.

about 40%. As a further control, livers were also removed from the same animals to test the microsomal desaturation activity. Since the results obtained were equivalent to those presented in Fig. 1, the data are not shown here.

The amount of arachidonic acid formed from labelled 20 : 3(n–6) incorporated in both groups of cells is compared in the lower panels. The general pattern of this curve was similar to that of the acid incorporation. The desaturation increased during the course of the experiment in both groups, but it was significantly lower in the diabetic adrenocortical cells at all the times tested. However, when the data from the diabetic cells were compared to those from the controls, it was evident that the inhibition observed was maximal at 30 min of incubation and remained throughout the remaining time points (right panel).

Effect of the addition of insulin to the incubation medium of isolated adrenocortical cells obtained from diabetic and control rats

A significant decrease in the incorporation of [1-^{14}C]eicosa-8,11,14-trienoic acid was seen in cells isolated from diabetic rats compared to those from control animals after 180 min of incubation (Fig. 4). Both parameters were partially restored when insulin was added to the incubation medium. No significant changes were observed after addition of the hormone to the medium of the cells isolated from control rats.

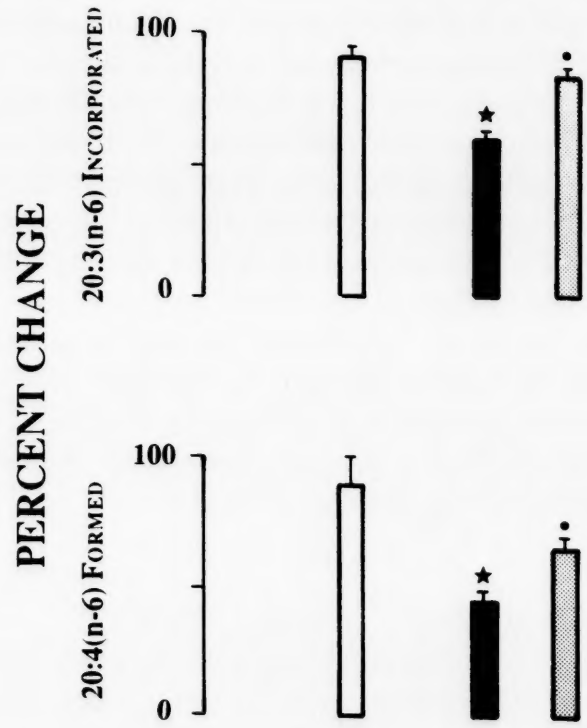

Fig. 4. Effect of the addition of insulin to the incubation medium on the incorporation (upper panel) and transformation (lower panel) of [1-^{14}C]eicosa-8,11,14-trienoic acid in isolated adrenocortical cells. Cells obtained from diabetic rats (filled columns), cells obtained from control rats (open columns) and from diabetic rats (hatched columns) incubated with insulin. Incubation time: 180 min. 100% corresponds to 2.830 $\pm$ 0.035 nmol/mg protein incorporated and 0.609 $\pm$ 0.016 nmol/mg protein transformed by non-hormone-treated control rats. The incubation conditions were as described in Materials and Methods. Values are the mean of four incubation flasks. Vertical lines represent $\pm$1 SEM. Symbols above columns indicate significant differences from control values (asterisk) and from diabetic values (open circle): $P < 0.001$.

Discussion

Diabetes mellitus is characterized by several metabolic changes that alter the utilization of glucose and fatty acids by the cells. Some of these changes were reflected in the enhancement of the plasma concentrations of glucose, triglycerides, total cholesterol and corticosterone.

STZ-treated animals displayed alterations in the biochemical parameters mentioned above that corresponded to those occurring in spontaneous diabetes and these abnormalities were reversed by insulin treatment (Table 1).

The results obtained in this work clearly show a significant depression in microsomal desaturation in the adrenal gland (Fig. 1). This inhibition was also indirectly indicated by the pattern of microsomal fatty acid composition, with an increase in the percentage of linoleic acid being accompanied by a marked decrease in that of arachidonic acid. These alterations were reflected in the $20:4(n-6)/18:2(n-6)$ ratio, with it showing a decrease of 41% in the diabetic state (Table 2). Indirect evidence for the activity of Δ^9-desaturase was obtained by analyzing the $18:1/18:0$ ratio, which parameter was reduced by approximately 43% in STZ-treated rats.

The behaviour of the desaturating activities in the adrenal gland observed in this work was similar to that showed in liver (Fig. 1), and these data were coincident with those extensively published and reviewed by Horrobin (1988).

In diabetic rats, the relative proportion of $20:4(n-6)$ acid decreased only 15% in contrast to the high increment observed in $18:2(n-6)$ acid (45%). This observation could be attributed to the specific characteristics of the fatty acid composition of the adrenal gland. In this tissue, the amount of arachidonic acid is 8-fold compared to that of linoleic acid (Table 2). For this reason, a comparable depression in the proportion of arachidonic acid would no doubt require a prolonged diabetic state in keeping with the slow turnover rate of this higher polyunsaturated fatty acid. The accumulation of $18:2(n-6)$ would be more evident because of the lesser amount of this acid present in the adrenal microsomes.

The adrenal gland is the tissue where the maximal accumulation of adrenoic acid ($22:4(n-6)$) was observed (Ostwald et al., 1964). Ten days of diabetes do not modify the relative percentages of adrenoic acid in the adrenal microsomes (Table 2). The strong tendency of this tissue to retain this acid could be related to the already known important role that adrenoic acid plays in the regulation of steroidogenesis (Igarashi and Kimura, 1986).

Polyunsaturated fatty acid metabolism in the adrenal gland and liver is subjected to hormonal control. In addition, both tissues respond in the same way to 'in vivo' or 'in vitro' treatment with a variety of hormones that have different structures and physiological actions (Gómez Dumm et al., 1976, 1979; Mandon et al., 1986, 1987). In the liver the activity of the desaturases is insulin-dependent. The results obtained in this work show that these enzymes are also modified by insulin treatment in the adrenal gland (Fig. 1). Nevertheless, the ability of insulin replacement to restore the activities of the desaturases was different in the liver and the adrenal gland of diabetic rats. In the former organ insulin treatment almost doubled the activities of Δ^6- and Δ^5-desaturases observed in the untreated diabetic rats. The recovery observed in the adrenal gland though less pronounced (Fig. 1) was nevertheless sufficient to restore the fatty acid composition to the control pattern (Table 2).

The formation of a thioester bond between a fatty acid and coenzyme A is the first step in the elongation and desaturation of polyunsaturated fatty acid biosynthesis. This reaction is catalyzed by a long chain fatty acyl-CoA synthetase and is a potentially important site of regulation of polyenoic fatty acid metabolism.

The inhibition produced by diabetes in the long chain fatty acyl-CoA synthetase for different substrates in liver and adrenal gland agrees with that observed by Saggerson and Carpenter (1987) in adipose tissue. Moreover, these data are consistent with the existence of a common enzyme for a wide range of long chain fatty acids in these tissues. A conclusion based on studies of kinetic parameters (Tanaka et al., 1979; Wilson et al., 1982; Mandon et al., 1988). Although this enzyme responds in the same way to ACTH and catecholamines (Sooranna and Saggerson, 1978; Hall and Saggerson, 1985; Mandon et al., 1988), its behaviour in the three tissues differs in the presence of insulin, with that

hormone partially restoring the enzymatic activity in adipose tissue (Saggerson and Carpenter, 1987) but having no such effect in either liver or adrenal gland (Fig. 2).

In the presence of saturating concentrations of substrate, the maximal desaturase activities of adrenal gland and liver in control rats were about 0.8 nmol/mg protein/min, while the activities of fatty acid acyl-CoA synthetase in these tissues were approximately 30 nmol/mg protein/min. Even after maximal depression of acyl-CoA synthetase in these tissues, in diabetic state, there still remained an enzymatic activity of approx. 10–20 nmol/mg protein/min. Upon analysis of the relationship between the activities of the desaturases and these acyl-CoA synthetases, we conclude that, even under the conditions of maximal depression produced by the diabetic state, the amount of the substrate available for the desaturases is still many times higher than that necessary for attaining substrate saturation.

From the results obtained in this work and the data previously reported (Mandon et al., 1988), we conclude that the inhibition of fatty acyl-CoA synthetase observed here in liver and adrenal microsomes cannot be responsible for the depression in polyunsaturated fatty acid desaturases produced under these metabolic conditions.

Research on biological phenomena in the whole adrenal gland 'in vivo' is often complicated because of the different types of cells that form this tissue. Therefore, in this portion of our studies we have restricted our investigation to cells isolated from the fasciculata-reticularis zone of the cortex since these elements are responsible for corticosterone secretion.

The strong inhibition of Δ^5 desaturation activity observed in the diabetic adrenal microsomes was also seen when the adrenocortical cells were isolated from the STZ-treated rats and incubated in the presence of 20 : 3(n–6) acid. After 30 min of incubation, a 60% inhibition in the biosynthesis of arachidonic acid was accompanied by a less prominent blockade in the uptake of substrate (Fig. 3). Although the former effect could be attributed in part to the depression in the incorporation of 20 : 3(n–6) acid and consequently to a lesser amount of the precursor available inside the cells, the blockade in desaturation was nevertheless higher than the inhibition in the incorporation of the substrate and remained approximately constant for the duration of the incubation. More importantly, we consider that the depression in Δ^5 desaturation activity could be responsible for the inhibition observed in 20 : 3(n–6) acid uptake. Only traces of this acid are present in the adrenocortical cells and could not, for example, be detected by studying the metabolization of labelled 18 : 2(n–6) acid (Mandon and Gómez Dumm, 1989), thus indicating that the most important metabolic function performed by the eicosatrienoic acid is to serve as the substrate for arachidonic acid biosynthesis. The depression in Δ^5 desaturation activity observed in the cells from the insulin-deprived animals was similar to that seen in the diabetic adrenal microsomes, where the activity of the enzyme was tested under conditions of substrate saturation.

Insulin added to the medium of adrenocortical cells isolated from diabetic rats partially restored arachidonic acid biosynthesis after 180 min of incubation. These results confirm that there is a direct cellular effect of the hormone, data that are consistent with the presence of insulin receptors in this type of cell as described earlier (Kitabchi and Sharma, 1971). This reversal was similar to that obtained in adrenal microsomes when the intact animals were injected with insulin. Another interesting observation within this experiment was that once Δ^5 desaturation activity became restored in the presence of insulin, the diminution in the incorporation of 20 : 3(n–6) acid was also reversed by a comparable amount; a quantitative result that, in fact, would have been predicted from the discussion of the preceding paragraph.

Based on the results obtained in the present experiments, we can conclude that the biosynthesis of polyunsaturated fatty acids in the adrenal gland was markedly affected by insulin deprivation. These abnormalities in fatty acid metabolism corresponded with those determined in the liver. The behaviour of the enzymatic activities resembles that observed when rats were injected with ACTH (Mandon et al., 1987). This similarity might suggest that the depression observed in the biosynthesis of polyunsaturated fatty acids could be the consequence of high levels of plasma ACTH

observed in diabetic rats (L'Age et al., 1974) and, in this regard, the diabetic state has already been seen to be accompanied by a hypersecretion of glucocorticoids. This observation, clearly shown in Table 1 and previously reported by other authors (De Nicola et al., 1976, 1977), has been attributed to stimulation by ACTH. Insulin treatment of diabetic rats both restored the activity of the desaturases and normalized plasma corticosterone levels. If the aforementioned interpretation were correct, we might assume that insulin restores these parameters indirectly through the regulation of ACTH secretion. However, large doses of insulin were unable to modify ACTH or corticosterone secretion in intact rats (Dallman et al., 1987). Moreover, the results obtained when insulin was added to the incubation medium of adrenocortical cells isolated from diabetic rats clearly demonstrated that this hormone exerts a direct effect on the restoration of arachidonic acid biosynthesis. On the basis of all of the above considerations, a more likely interpretation of the results presented here would be that insulin replacement in intact diabetic animals exerts its normalizing effects on hepatic and adrenal polyunsaturated fatty acid metabolism through direct hormonal action on those tissues themselves at the cellular level.

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Calendar of Meetings

Prostaglandins, Leukotrienes, Lipoxins & PAF
13–17 May 1991, Washington, DC, U.S.A.
Information: John Franklin (coordinator) and Mei Mei Gillespie, The Lisner Auditorium and the Marvin Center of The George Washington University, 21st and H Streets, NW, Washington, DC, U.S.A. Tel. (202) 994-3539 or 994-3709. Fax (202) 994-8964.

Endocrinology of Puberty
23–24 May 1991, Paris, France
Information: Scientific Secretariat, Dr. G. Copinschi or Dr. A. Caufriez, Laboratoire de Médecine Expérimentale, Faculté de Médecine, U.L.B., 2 rue Evers, B-1000 Brussels, Belgium.

10th International Symposium of the Journal of Steroid Biochemistry and Molecular Biology
26–29 May 1991, Paris, France
Information: Dr. J.R. Pasqualini, C.N.R.S. Steroid Hormone Research Unit, Foundation for Hormone Research, 26 Blvd Brune, 75014 Paris, France. Tel. (33) (1) 45.39.91.09; Fax (33) (1) 45.42.61.21.

73rd Annual Meeting of the Endocrine Society
19–22 June 1991, Washington, DC, U.S.A.
Information: Ann Singer, Meetings Manager, The Endocrine Society, 9650 Rockville Pike, Bethesda, MD 20814, U.S.A. Tel. 301-571-1802; Fax 301-571-1869.

International Thyroid Meeting
4–5 July 1991, Newcastle upon Tyne, U.K.
Information: Dr. P. Perros, Department of Medicine, Medical School, Newcastle upon Tyne NE2 4HH, U.K.

15th International Congress of Biochemistry
4–9 August 1991, Jerusalem, Israel
Information: 15th IUB Congress, KENES Ltd., P.O. Box 50006, Tel-Aviv 61500, Israel, Tel. 972-(3)-654571.

5th Meeting of the European Neuroendocrine Association (ENEA)
25–28 August 1991, Budapest, Hungary
Information: Congress Secretariat, 5th Meeting of the ENEA, P.O. Box 67, H-1450 Budapest 9, Hungary. Tel. 36-1-133-4538; Fax 36-1-114-1866.

19th Annual Meeting of the European Thyroid Association
25–31 August 1991, Hannover, F.R.G.
Information: Seminar-Congress-Service, Bernd Staupendahl GmbH, Postfach 51 04 07, 3000 Hannover, F.R.G. Tel. (0511) 54 80 30/50. Fax (0511) 5 49 82 16.

1st World Congress of Cellular and Molecular Biology
1–7 September 1991, Paris, France
Information: Mrs. Leila Orbecchi, Director C.E.R.T., 63 Avenue Parmentier, 71 011 Paris, France. Tel. (1) 48 07 07 00. Fax (1) 48 07 22 11.

36th Harden Conference on GTP-Binding Proteins
8–13 September 1991, Wye College, Ashford, U.K.
Information: Meeting Officer, The Biochemical Society, 59 Portland Place, London W1N 3AJ, U.K. Tel. 071-580.5530. Fax 071-323.1136.

4th International Congress on Hormones and Cancer
15–19 September 1991, Amsterdam, The Netherlands
Information: Congress Secretariat, Fourth International Congress on Hormones and Cancer, c/o RAI Organisatie Bureau Amsterdam bv, Europaplein 12, 1078 GZ Amsterdam, The Netherlands

2nd International Conference on Eicosanoids and other Bioactive Lipids in Cancer, Inflammation and Radiation Injury
18–21 September 1991, Berlin, F.R.G.
Information: Dr. Santosh Nigam, Eicosanoid Re-

search, Department of Gynecological Endocrinology, Universitätsklinikum Steglitz, Free University Berlin, Hindenburgdamm 30, D-1000 Berlin 45, F.R.G.

16th European Symposium Hormones and Cell Regulation
23–26 September 1991, Monte Ste. Odile, Alsace, France
Information: Dr. B. Hamprecht, Physiologisch-chemisches Institut der Universität, Hoppe-Seyler-Str. 4, D-7400 Tübingen, F.R.G. Fax 49-7071-293361.

International Symposium on Recent Advances in Mammalian Pheromone Research
6–9 October 1991, Paris, France
Information: Dr. David L. Berliner, EROX Corporation, 535 Middlefield Road, 240, Menlo Park, CA, U.S.A. Tel. (1) (415) 324.1231. Fax (1) (415) 324.2819; or: Dr. Jorge R. Pasqualini, Steroid Hormone Research Unit, Foundation for Hormone Research, 26 Bouelvard Brune, 75014 Paris, France. Tel. (33) (1) 45.39.91.09. Fax (33) (1) 45.42.61.21.

Endocrine Society – 43rd Postgraduate Assembly Meeting
9–13 October 1991, San Diego, CA, U.S.A.
Information: Ann Singer, Meetings Manager, The Endocrine Society, 9650 Rockville Pike, Bethesda, MD 20814, U.S.A. Tel. 301-571-1802; Fax 301-571-1869.

International Symposium on A Molecular View of Steroid Biosynthesis and Metabolism
14–17 October 1991, Jerusalem, Israel
Information: Dr. Israel Hanukoglu, Department of Hormone Research, Weizmann Institute of Science, Rehovot 76100, Israel.

8th International Conference on Second Messengers and Phosphoproteins — The Biology and Medicine of Signal Transduction
3–8 August 1992, Glasgow, Scotland, U.K.
Information: Dr. P.R.M. Dobson, General Secretary, 8th International Conference on Second Messengers and Phosphoproteins, Institute of Endocrinology (HMCB), University of Sheffield Medical School, Beech Hill Road, Sheffield S10 2RX, U.K.

9th Meeting of the International Society of Endocrinology
30 August–5 September 1992, Nice, France
Information: Chairman Local Organizing Committee, Dr. R. Mornex, Hôpital Edouard Herriot, Place d'Arsonval, 69437 Lyon Cedex 08, France. Tel. 7234 4815; Fax 7234 8476. Further details: Secretary General, Professor Lesley H. Rees, Department of Chemical Endocrinology, 51–53 Bartholomew Close, London EC1A 7BE, U.K.

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Announcement

16th European Symposium Hormones and Cell Regulation

23–26 September 1991, Mont Ste. Odile, Alsace, France

Topics: nitric oxide and cyclic nucleotides; factors in the development of the nervous system; hormonal factors regulating embryonic development; GTP-binding proteins; receptors and channels; role of protein kinases and phosphoprotein phosphatases in signal transduction.

Meeting format: invited lectures and posters.

Information: Dr. B. Hamprecht, Physiologisch-chemisches Institut der Universität, Hoppe-Seyler-Str. 4, D-7400 Tübingen, F.R.G., Telefax: 49-7071-293361.

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Rapid Paper

Progesterone acts at the plasma membrane of human sperm

Stanley Meizel and Kenneth O. Turner

Department of Cell Biology and Human Anatomy, School of Medicine, University of California – Davis, Davis, CA 95616-8643, U.S.A.

(Received 15 January 1991; accepted 18 February 1991)

Key words: Progesterone; Steroid; Sperm; Plasma membrane; Calcium; Acrosome reaction; (Human)

Summary

There has been increasing interest in the relationship between rapid effects of steroids and steroid–plasma membrane interaction. This laboratory has previously reported that progesterone increases human sperm cytosolic free calcium ($[Ca^{2+}]_i$) and thereby initiates the human sperm acrosome reaction (AR) in < 1 min. Herein, to test whether progesterone acts at the sperm plasma membrane, progesterone 3-(*O*-carboxymethyl)oxime : bovine serum albumin (BSA) conjugate (free of unconjugated progesterone) was added to capacitated human sperm. Fura-2 assays were used to detect < 1 min changes in $[Ca^{2+}]_i$, and indirect immunofluorescence was used to assay the AR occurring 1 min after stimulus addition. The conjugate increased $[Ca^{2+}]_i$ and the AR (though less than did unconjugated progesterone). Enzyme immunoassays demonstrated that the concentrations of unconjugated progesterone in conjugate-treated sperm suspensions did not increase over those of control suspensions. Since the progesterone : BSA conjugate presumably does not cross the sperm plasma membrane, progesterone must act at that membrane to increase $[Ca^{2+}]_i$ and the AR.

Introduction

The mammalian sperm acrosome reaction (AR) is a specialized exocytotic process involving fusion of a membrane of the acrosome (a secretory granule-like organelle in the sperm head) with the overlying sperm plasma membrane (see discussion by Yudin et al., 1988). The mammalian AR is essential for fertilization and occurs, in the presence of an initiator, in sperm that have been previously modified in some way (capacitated) by residence in the female reproductive tract or by in vitro incubation (Yanagimachi, 1988). The human AR can be initiated in vitro by at least two molecules present at the site of fertilization, an egg zona pellucida component (Cross et al., 1988) and progesterone (Osman et al., 1989).

Progesterone and several other progestins, but not other steroids tested, rapidly increase cytosolic free calcium ($[Ca^{2+}]_i$) in uncapacitated and capacitated human sperm (Thomas and Meizel, 1989; Blackmore et al., 1990; Meizel et al., 1990). Through this increased $[Ca^{2+}]_i$, progesterone initiates the AR in capacitated human sperm within 1 min after addition (Meizel et al., 1990). The lowest effective concentration for AR initiation (under a particular set of in vitro conditions), 250 ng/ml, is much higher than blood levels, but progesterone may be present in μg/ml concentra-

Address for correspondence: Dr. Stanley Meizel, Department of Cell Biology and Human Anatomy, School of Medicine, University of California – Davis, Davis, CA 95616-8643, U.S.A.

tions at the fertilization site (Osman et al., 1989; Meizel et al., 1990).

There is increasing interest in the relationship between rapid, non-genomic cellular effects of steroids and possible steroid interactions with the plasma membrane (Koenig et al., 1989; Schumacher, 1990). The rapid responses of human sperm to progesterone suggest steroid action at the plasma membrane (Thomas and Meizel, 1989), particularly since the mature sperm lacks the cytoplasmic apparatus for protein synthesis, but the possibility still exists that the steroid must enter the cytoplasm to exert its effects. In the present report, the hypothesis that progesterone acts at the plasma membrane was tested by determining whether a progesterone covalently bound to bovine serum albumin (BSA) would rapidly increase $[Ca^{2+}]_i$ and initiate the AR in capacitated human sperm. Since the progesterone : BSA conjugate presumably would not rapidly diffuse across the sperm plasma membrane, and since sperm are not known to exhibit endocytotic activity, any effects of that conjugate should be due to the steroid's action at the sperm plasma membrane. Part of these results were presented elsewhere as an abstract (Meizel and Turner, 1990).

Materials and methods

Removal of unconjugated progesterone from the progesterone:BSA conjugate. Progesterone 3-(*O*-carboxymethyl)oxime:BSA (31 mol progesterone/mol BSA, Sigma Chemical Co., St. Louis, MO, U.S.A.) was dissolved in phosphate-buffered saline (PBS) (5.5 mg in 5 ml) into which 3 mg Norite and 0.3 mg dextran were then added. The resulting mixture was gently stirred for 24 h at 4°C and then centrifuged twice at 48,000 × *g* for 90 min at 2°C. The protein content of the conjugate supernatant was determined using $E_{1\%}$ at 279 nm = 6.67 (Janatova et al., 1968). Aliquots were stored at −21°C. Stock solutions were 1.24 mg and 0.124 mg per ml PBS.

Preparation and capacitation of sperm. Human semen was obtained by masturbation from healthy donors. A > 95% motile population of sperm was prepared using a discontinuous Percoll gradient (Suarez et al., 1986). Washed sperm were resuspended (6×10^6 sperm/ml) in a modified Tyrodes solution (Thomas and Meizel, 1988) that included 26 mg BSA/ml (catalogue No. 7030, Sigma Chemical Co., St. Louis, MO, U.S.A.). The resuspended sperm were then capacitated by incubating 200 μl aliquots in 15 ml polystyrene conical centrifuge tubes (Sarstedt, Hayward, U.S.A.) at 37°C for 24 h for AR studies or at 40°C for 6.5 h for fura-2 studies (Thomas and Meizel, 1988) in a humid atmosphere of 5% CO_2-95% air (pH of media, 7.4–7.6).

Assay of the effect of the progesterone : BSA conjugate on AR initiation. In each experiment, the following were added to duplicate 200 μl aliquots of sperm suspensions (concentrations are final in sperm suspensions): 52 μg conjugate/ml PBS (equivalent to 6.66 μg of progesterone/ml) or 5.2 μg conjugate/ml PBS (equivalent to 0.66 μg progesterone/ml) or 0.66 μg progesterone/ml progesterone solvent medium or a solvent control. The stock solution of progesterone added consisted of 100 μg/ml progesterone and 10% dimethyl sulfoxide (DMSO) (v/v) dissolved in a Hepes buffered medium (Thomas and Meizel, 1989) containing 26 mg/ml BSA (catalogue No. A7030, Sigma Chemical Co., St. Louis, MO, U.S.A.). The same volume of PBS used for conjugate additions was added to progesterone and solvent control tubes, and the same volume of progesterone solvent medium used for progesterone additions was added to the conjugate and solvent control tubes. In all incubation suspension tubes, the final concentrations of DMSO and A7030 BSA were 0.1% (v/v) and 26 mg/ml respectively. Sperm motility and quality was evaluated by phase contrast microscopy of 10 μl of suspensions (Suarez et al., 1986). The remaining 190 μl aliquot of each type of treated sperm suspension was fixed without washing, one min after additions and the AR determined by indirect immunofluorescence using mouse monoclonal HS-21, a gift of Dr. K. Bechtol (Suarez et al., 1986; Thomas et al., 1988). Duplicate aliquots were used for assay of progesterone (see below).

Assays for unconjugated progesterone. Aliquots (200 μl) of the capacitation medium and the conjugate in that medium or of capacitated sperm suspensions 1 min after conjugate or solvent control additions were extracted with 2 ml of petroleum ether. After solvent evaporation, residues

were assayed for progesterone with a solid-phase, microtiter plate, enzyme immunoassay procedure (Munro and Stabenfeldt, 1984) by the U.C. Davis Vet. School Clinical Endocrinology Lab.

Assay of the effect of progesterone: BSA conjugate on $[Ca^{2+}]_i$. The methods for determining $[Ca^{2+}]_i$ using fura-2 were essentially those used by Thomas and Meizel (1988, 1989). In brief: capacitated sperm resuspended in a Hepes-buffered medium without glucose were incubated for 30 min with 1 μM of the acetoxymethyl ester of fura-2 (Molecular Probes, Junction City, OR, U.S.A.) and centrifuged through 40% Percoll to remove extracellular fura-2; additions of conjugate, progesterone or solvent control to 1 ml aliquots of fura-2 loaded sperm suspensions (6×10^6/ml) were made in a stirred cuvette at 40°C; spectrofluorometric assays used a single wave length of excitation (339 nm) with emission monitored at 500 nm; estimation of $[Ca^{2+}]_i$ was made according to Pollock et al. (1986), assuming a K_d of 224 nM for Ca^{2+}/fura-2 (Grynkiewicz et al., 1985). The calibration of the fura-2 signal was modified by substituting the optimal concentration of ionomycin (calcium salt, Calbiochem, La Jolla, CA, U.S.A.), 16 μM (established by titration), for digitonin in determining Ca^{2+}-saturated fura-2 fluorescence.

Materials. Other chemicals were reagent grade and were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.) or Fisher Scientific Co. (Fair Lawn, NJ, U.S.A.).

Results

Unconjugated progesterone was detected in untreated conjugate (32 ng/μg) but not in the capacitation medium or in dextran-charcoal treated conjugate. Dextran-charcoal treated conjugate used at the equivalent of 0.66 and 6.6 μg of progesterone/ml significantly increased the AR over the solvent control (Table 1). However, both concentrations of conjugate were less effective than unconjugated progesterone at 0.66 μg/ml (Table 1). The very low levels of unconjugated progesterone present in the conjugate-treated sperm suspensions did not differ significantly from that present in solvent control suspensions during the 1 min incubation of conjugate with sperm (Table 1).

The BSA used to prepare the conjugate was Sigma catalogue No. A7888 (Sigma Chemical Co. Technical Service). In preliminary experiments, addition to capacitated human sperm of 5.2 and

TABLE 1

ACROSOME REACTION OF HUMAN SPERM AND OF THE CONCENTRATION OF UNCONJUGATED PROGESTERONE IN SPERM SUSPENSIONS 1 MIN AFTER ADDITION OF PROGESTERONE: BSA CONJUGATE

Treatment ($n = 6$) [a]	% AR (mean ± SD)	ng unconjugated progesterone/ml sperm suspension (mean ± SD)
Progesterone (0.66 μg/ml)	44.83 ± 5.13 [b]	–
Conjugate (52 μg/ml = 6.6 μg/ml progesterone) [e]	27.58 ± 7.28 [b,c]	1.16 ± 0.33 [d]
Conjugate (5.2 μg protein/ml = 0.66 μg/ml progesterone) [e]	29.32 ± 5.03 [b,c]	1.11 ± 0.33 [d]
Solvent control [f]	14.95 ± 4.63	1.37 ± 0.45

[a] Sperm motility was 80–90% in all experiments, and there was no difference in percent motility or the intensity of motility between control and experimental treatments.

[b] Significant difference in AR between treatment and solvent control, $p < 0.05$ by Dunnett t-test for multiple comparisons (percentage data transformed by arcsin $\sqrt{p}$).

[c] Significant difference in AR between conjugate and unconjugated progesterone, $p < 0.05$ by Dunnett t-test for multiple comparisons (percentage data transformed by arcsin $\sqrt{p}$).

[d] No significant difference between treatment and solvent control, $p \geq 0.15$ by paired t-test with Bonferroni correction for multiple comparisons.

[e] Based on 31 mol of progesterone/mol of BSA (Sigma Chemical Co. Technical Service).

[f] See Materials and Methods for solvent control.

Fig. 1. The effect of the progesterone:BSA conjugate on the $[Ca^{2+}]_i$ of fura-2 loaded capacitated human sperm. Traces are representative of three similar experiments (sperm motility was 85%): (a) untreated sperm; (b) sperm treated with the dextran-charcoal preparation of BSA (in PBS) used to synthesize conjugate; (c) sperm treated with conjugate (5.2 μg protein/ml = 0.66 μg/ml progesterone) in PBS; (d) sperm treated with 0.66 μg/ml progesterone. Solvent for progesterone (see Materials and Methods) has no effect on the $[Ca^{2+}]_i$ (Thomas and Meizel, 1989). All traces were corrected for the presence of extracellular fura-2 (Thomas and Meizel, 1988).

52 μg/ml of a dextran-charcoal treated preparation of A7888 BSA did not initiate the AR (data not shown). Thus, dextran-charcoal treated A7888 BSA was not included in the controls for experiments shown in Table 1.

The conjugate (but not dextran-charcoal treated A7888 BSA) stimulated a rapid, transient increase in sperm $[Ca^{2+}]_i$, though less than did progesterone (Fig. 1).

Discussion

Steroid-synthetic polymer conjugates have been previously used to demonstrate that progesterone agonists can act at the plasma membrane to stimulate maturation of *Xenopus laevis* oocytes (Ishikawa et al., 1977; Baulieu et al., 1978), a cell which appears to have a plasma membrane progesterone receptor (Sadler and Maller, 1982). Progesterone : BSA conjugates have been used to study putative progesterone receptors in rat brain nerve cell membrane preparations (Ke and Ramirez, 1990). The present studies utilizing a progesterone : BSA conjugate demonstrate that progesterone, even when covalently-bound to a large molecule which presumably does not cross the sperm plasma membrane, will rapidly increase $[Ca^{2+}]_i$ and initiate the AR in capacitated human sperm. It is unlikely that unconjugated progesterone, released in some way from the conjugate, is responsible for the present results because the very low concentrations of unconjugated progesterone present in conjugate-treated sperm suspensions were not different than those of control suspensions during the 1 min incubations. The lowered effectiveness of the conjugate compared to unconjugated progesterone could be due to the modification of progesterone at the C-3 position in the former or to some of the covalently-bound progesterone being present at sites in the BSA that are not readily accessible to interaction with the sperm membrane.

The present results support the hypothesis that progesterone is acting at the sperm plasma membrane rather than having to enter the cytoplasm. Rapid effects and specific plasma membrane binding sites have been reported for various steroids in several cell types (Schumacher, 1990). Plasma membrane binding sites for progesterone in human sperm have been reported (Hyne and Boettcher, 1977; Cheng et al., 1981), and a preliminary report described the same progesterone-binding protein present in both human seminal plasma and sperm (Neulen et al., 1990). However, an integral sperm plasma membrane protein with high specificity and high affinity for progesterone has not yet been demonstrated. Whether progesterone increases the $[Ca^{2+}]_i$ and the AR of human sperm by interaction with a specific plasma membrane receptor and/or through some other mechanism(s) at the plasma membrane (Meizel et al., 1990) remains to be determined.

Acknowledgements

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Rapid Paper

Differences in LH receptor down-regulation between rat and mouse Leydig cells: effects of 3′,5′-cyclic AMP and phorbol esters

Anthony P. West, M. Pilar Lopez-Ruiz and Brian A. Cooke

Department of Biochemistry, Royal Free Hospital School of Medicine, Rowland Hill Street, London, NW3 2PF, U.K.

(Received 25 February 1991; accepted 7 March 1991)

Key words: Receptor; Lutropin; Cyclic AMP; Phorbol ester; Leydig cell; Down-regulation

Summary

The role of cyclic AMP and phorbol esters in luteinizing hormone (LH) receptor down-regulation in Leydig cells has been studied. Dibutyryl cyclic AMP (db-cAMP) (0.01, 0.1 and 1 mM), forskolin (80 μM) and cholera toxin (1.19 nM) caused a 30–50% loss of [^{125}I]hCG binding sites and an inhibition of receptor-[^{125}I]hCG complex internalization in mouse tumour Leydig (MA10, MLTC-1) cells during 2 h. In contrast, db-cAMP had no effect on the level of binding sites or internalization of the hormone receptor complex in rat testis Leydig cells or a rat tumour (R2C) Leydig cell. Phorbol 12-myristate 13-acetate (PMA) at concentrations from 10^{-9} to 10^{-5} M had no effect on hormone binding or hormone-receptor complex internalization in any of the Leydig cells. In contrast a 2 h preincubation of MLTC-1 cells with 10^{-7} M PMA caused a loss of subsequent LH-stimulated cyclic AMP and pregnenolone production. These results indicate that LH receptor down-regulation is mediated by cyclic AMP dependent kinase, but not protein kinase C, in mouse Leydig cells. No down-regulation of rat Leydig cell LH receptor occurs with either kinase.

Introduction

The luteinizing hormone (LH) receptor exists in a dynamic state exhibiting recycling in the rat testis Leydig cell (Habberfield et al., 1987) and continual receptor synthesis in the cultured mouse tumour (MA10) Leydig cell (West et al., 1990). In mouse Leydig cells (Freeman and Ascoli, 1981; Lefèvre et al., 1985) the LH receptor exhibits ligand-induced loss, i.e. down-regulation, of cell surface binding capacity when the cells are desensitized. This negative modulation of LH receptors is not observed in rat Leydig cells during desensitization. At concentrations of LH that cause down-regulation of the LH receptor in mouse Leydig cell, proteolytic cleavage of the hormone-receptor complex occurs at the plasma membrane, which is due to an inhibition of the internalization mechanism (West and Cooke, 1991). In this study we present evidence to show that the down-regulation of LH receptors is dependent on cyclic AMP and not PMA in mouse Leydig cells.

Address for correspondence: Anthony P. West, Department of Biochemistry, Royal Free Hospital School of Medicine, Rowland Hill Street, London, NW3 2PF, U.K.

Materials and methods

Ovine LH (batch oLH-26, potency: 2.3 U/mg) and hCG (batch CR-127, potency: 14,900 IU/mg)

were obtained from the National Institute of Child Health and Human Development (Bethesda, U.S.A.). Crude hCG (5000 IU) was obtained from Serono Laboratories (Hertfordshire, U.K.). Phorbol 12-myristate 13-acetate (PMA) and dibutyryl cyclic 3′,5′-adenosinemonophosphate (db-cAMP) were purchased from the Sigma Chemical Company (Dorset, U.K.). Powdered media and sera were obtained from Gibco (Middlesex, U.K.). Carrier free Na ^{125}I was obtained from the Radiochemical Centre, Amersham (Amersham, U.K.). All other reagents were purchased from Sigma or BDH (Dorset, U.K.).

Stock cultures of MA10, MLTC-1 and R2C cells were maintained according to the method of Ascoli (1981). Rat testis Leydig (RTL) cells were isolated and purified from adult male Sprague-Dawley rats (200–250 g) under non-sterile conditions, as described by Platts et al. (1988). Viability and purity of the cells were determined by the trypan blue exclusion method (Jeejeebhoy et al., 1975), diaphorase histochemistry (Aldred and Cooke, 1983) and by the 3β-hydroxysteroid dehydrogenase assay (Cooke et al., 1983). Iodination of hCG and [^{125}I] hCG binding were performed as previously described (West and Cooke, 1991). Internalization of [^{125}I]hCG was performed as indicated in the legend for Fig. 2. Pregnenolone and cyclic AMP were measured according to the methods of Van der Vusse et al. (1975), Steiner et al. (1972) and Harper and Brooker (1975).

Results

Dix et al. (1982, 1987) reported that PMA, but not cyclic AMP can cause desensitization in rat Leydig cells. Fig. 1 shows the effect of preincubating MLTC-1 cells (similar results were obtained for MA10 cells) for 2 h with either LH (3.3 nM), db-cAMP (1 mM) or PMA (10^{-7} M) on subsequent LH-stimulated pregnenolone and cyclic AMP production. After preincubation, the cells were stimulated for 2 h with or without LH (3.3 nM). Pretreatment with LH or PMA markedly decreased the ability of LH to stimulate cyclic AMP production (Fig. 1*A*). Further stimulation of pregnenolone production was inhibited by pretreatment with LH, db-cAMP or PMA (Fig. 1*B*). Basal pregnenolone production in cells pretreated

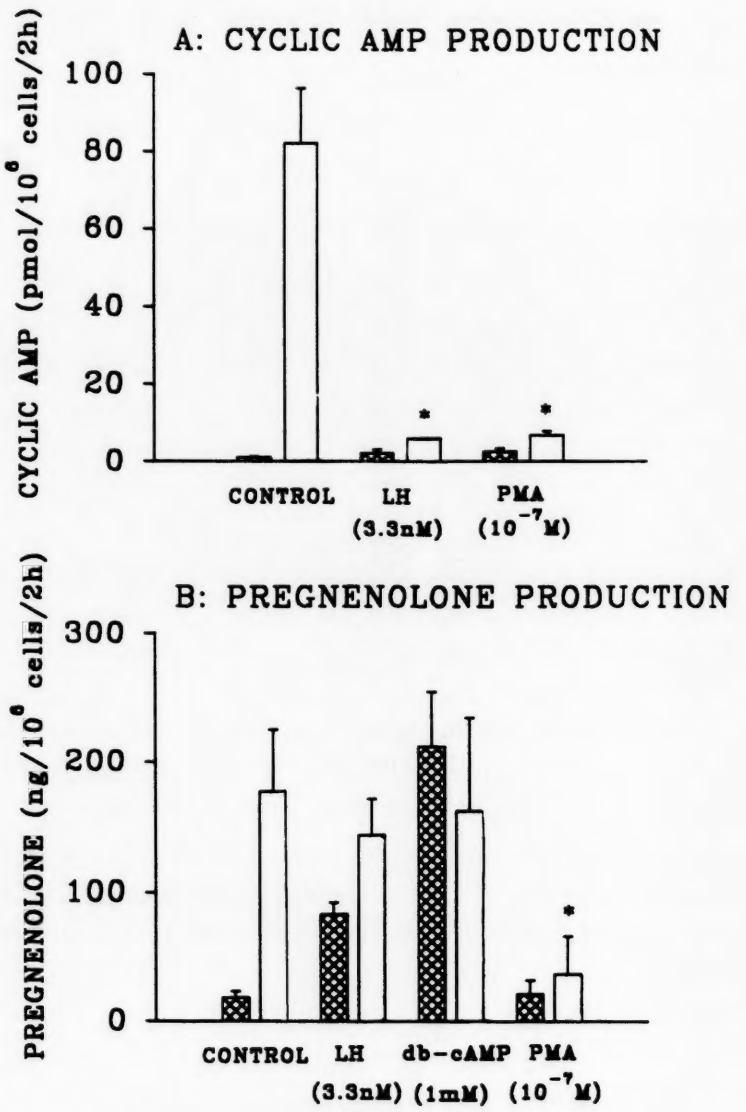

Fig. 1. The effect of LH, db-cAMP and PMA pretreatment on subsequent pregnenolone and cyclic AMP production. MLTC-1 cells were plated at 150×10^3 cells/well in 1 ml medium. The cells were preincubated for 2 h in the absence or presence of LH (3.3 nM), db-cAMP (1 mM) or PMA (10^{-7} M). Medium was removed and cells were washed with glycine-buffered saline (pH 3.0) to remove excess hormone. Cells were then incubated for 2 h in the absence (cross-hatched columns) or presence of 3.3 nM LH (open columns). Incubations were stopped with $HClO_4$ and stored frozen at $-20\,^{\circ}C$. Samples were thawed and neutralized with K_3PO_4 prior to radioimmunoassay of cyclic AMP (*A*) and pregnenolone (*B*) (values given are the mean $\pm$SD, $n = 2$, replicates = 3, $P < 0.01$, paired Student *t*-test).

with LH or db-cAMP was significantly higher than in control cells.

To study the levels of LH receptors during desensitization, mouse and rat Leydig cells were incubated for 2 h at 34°C, with db-cAMP (0.01, 0.1 and 1 mM) or PMA (10^{-9} to 10^{-5} M). Db-cAMP caused a 30–50% loss in detectable

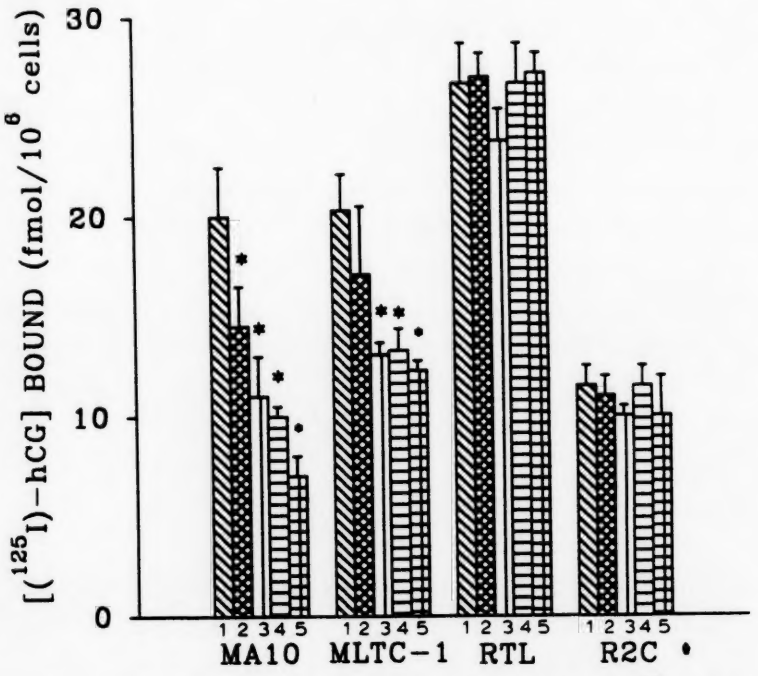

Fig. 2. The effect of db-cAMP on the levels of [^{125}I]hCG binding sites in Leydig cells. Leydig cells were plated at 150×10^3 cells/well in 1 ml medium. The cells were incubated for 2 h in the absence (1) or presence of 0.01 mM (2), 0.1 mM (3), 1 mM db-cAMP (4) or 3.3 nM (5) LH. At the times indicated cells were placed on ice and washed twice with 0.01 M PBS-BSA before [^{125}I]hCG binding was determined (values given are the mean ± SD, $n = 2$, replicates = 6).

[^{125}I]hCG binding sites by 120 min in MA10 and MLTC-1 cells, with no effect on binding sites in rat Leydig and R2C cells (Fig. 2). PMA had no significant effect on binding sites, by 120 min, in any of the Leydig cells (Table 1). Treatment with db-cAMP or PMA had no effect on the dissociation constant for [^{125}I]hCG in Leydig cells (data not shown).

TABLE 1

THE EFFECT OF PMA ON THE LEVELS OF [^{125}I]hCG BINDING SITES IN LEYDIG CELLS

Leydig cells were plated at 150×10^3 cells/well in 1 ml growth medium. Rat Leydig cells were preincubated for 2 h. The cells were incubated for 2 h with PMA at concentrations from 10^{-9} to 10^{-5} M. After 2 h the cells were placed on ice and washed twice with 0.01 M PBS-BSA before [^{125}I]hCG binding was determined (values given are the mean ± SD, $n = 2$, replicates = 6).

PMA (M)	Binding sites (fmol/10^6 cells)			
	MA10	MLTC-1	RTL	R2C
0	10.7±2.11	19.5±1.2	26.7±1.97	21.2±4.9
10^{-9}	7.9±2.11	20.8±4.3	26.0±2.8	14.8±4.2
10^{-8}	6.3±1.3	16.2±3.5	28.0±2.4	17.0±7.2
10^{-7}	6.6±2.0	18.6±2.6	27.1±3.3	28.4±9.1
10^{-6}	12.6±0.6	20.8±4.7	20.3±3.6	17.0±5.6
10^{-5}	9.2±1.3	16.7±6.1	23.9±2.55	23.9±7.6

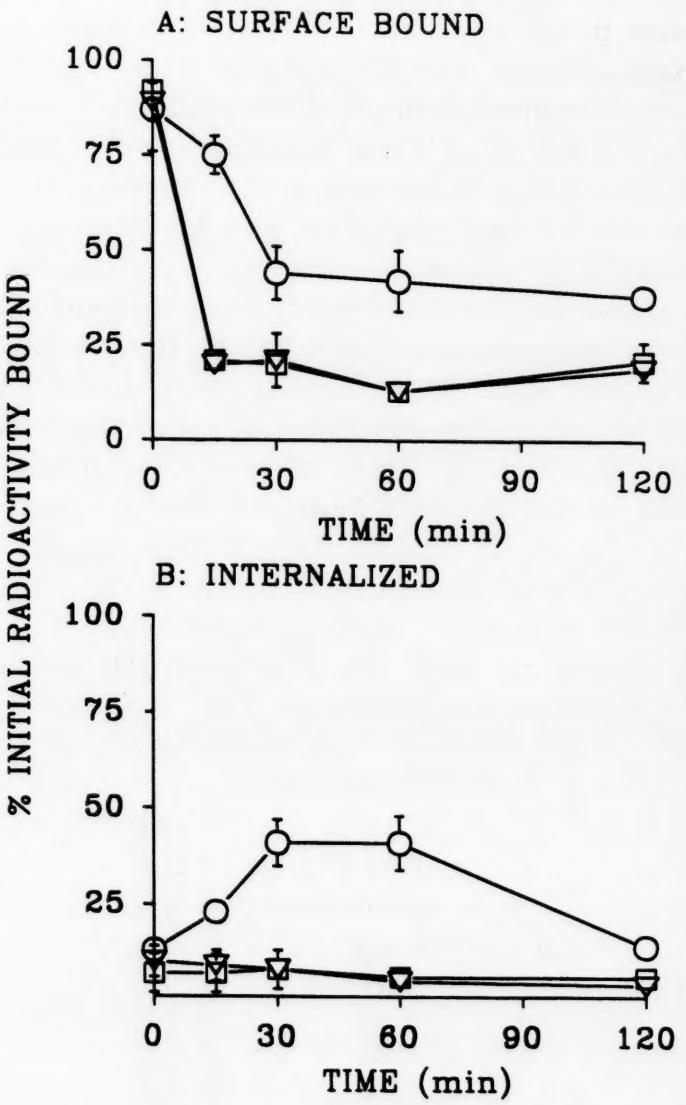

Fig. 3. The effect of db-cAMP and PMA on the internalization of [^{125}I]hCG in MA10 cells. Cells were plated at 2×10^6/well in 6-well plates. Two days later the cells were incubated at 10 °C, to inhibit internalization (Habberfield et al., 1987), in 1 ml fresh growth medium with 1.4 nM [^{125}I]hCG (○) alone or with either 0.1 (▽) or 1 mM (□) db-cAMP for 1 h. The cells were then placed on ice and washed with 0.01 M PBS-BSA to remove unbound hormone. The cells were then recultured in 1 ml fresh growth medium at 34 °C with the same additions as before, but excluding the [^{125}I]hCG. At the times indicated the plates were placed on ice, medium removed and washed with 0.01 M PBS-BSA. The surface bound [^{125}I]hCG (A) was determined by washing the cells twice with 50 mM glycine-buffered saline (pH3) (Ascoli, 1982) and counting the combined washings. To measure the amount of internalized hormone (B), cells were dissolved in 1 ml of 0.5 M NaOH, and counted in a γ-counter (values represent the mean ± SD, $n = 2$, replicates = 2).

We have previously shown that the mechanism for the down-regulation of LH receptors in MA10 cells is a result of an inhibition of internalization allowing proteolytic cleavage of the receptor at the plasma membrane (West and Cooke, 1990). We therefore studied the profile of receptor internalization in the presence of db-cAMP or PMA and investigated the possibility of proteolytic cleavage being involved in db-cAMP-induced down-regulation. Fig. 3 shows the effect of db-cAMP on the internalization of [^{125}I]hCG in MA10 cells. Surface associated radioactivity (Fig. 3*A*) in the presence of db-cAMP demonstrated a more rapid decline than in control cells, though there was no increase in internalized levels (Fig. 3*B*). A similar profile was obtained for MLTC-1 cells. Incubating MA10 and MLTC-1 cells with PMA or rat Leydig cells with db-cAMP or PMA showed no significant effect on the internalization profile of [^{125}I]hCG (data now shown). Fig. 4 shows the effect of preincubating MA10 cells in the presence of protease inhibitors (leupeptin 100 μM, phenylmethylsulphonyl fluoride (PMSF) 10 μM, aprotinin 900 units/ml) for 30 min before the addition of reagents. The cells were further incubated for 2 h with LH (3.3 nM), cholera toxin (1.19 nM), or forskolin (80 μM). Cholera toxin and forskolin induced a 50–70% loss in binding sites. Incubating the cells in the presence of protease inhibitors prevented any loss in binding sites.

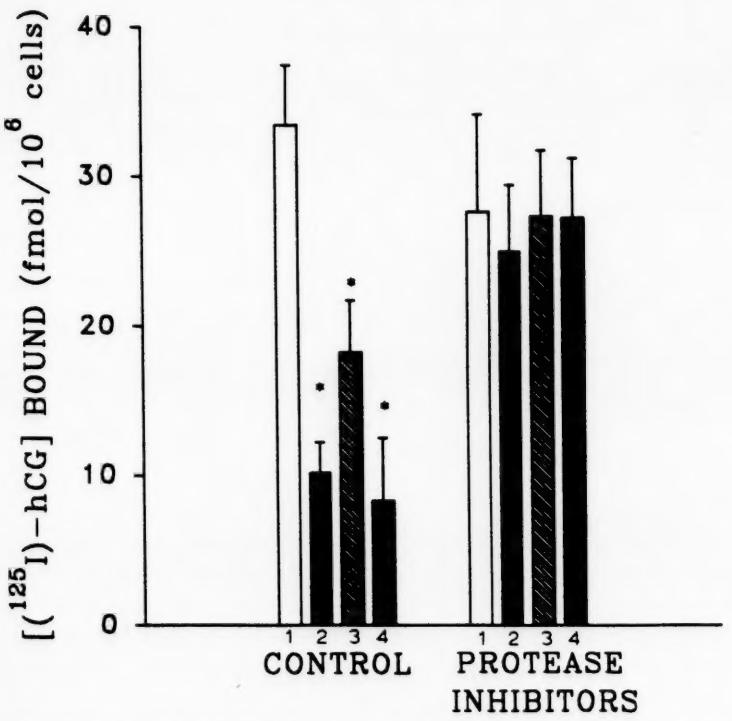

Fig. 4. The effect of protease inhibitors on the levels of [^{125}I]hCG binding sites in Leydig cells. MA10 cells were plated at 150×10^3 in 1 ml medium and preincubated ± protease inhibitors (leupeptin 100 μM, PMSF 10 μM, aprotinin 900 units/ml) in 1 ml fresh medium, for 30 min at 34° C. The cells were further incubated ± protease inhibitors for 2 h either in the absence (1) or presence of 3.3 nM LH (2), 1.19 nM cholera toxin (3), or 80 μM forskolin (4). After 2 h the cells were placed on ice and washed twice with 50 mM glycine-buffered saline (pH 3.0) before [^{125}I]hCG binding was determined (values given are the mean ± SD, $n = 2$, replicates = 6, $^*P < 0.05$, paired Student t-test).

Discussion

Previously, we have reported that LH causes down-regulation in mouse Leydig cells by inhibiting internalization followed by proteolytic cleavage of the extracellular fragment of the LH receptor (West and Cooke, 1991). The present study demonstrates that this is a cyclic AMP-dependent process because db-cAMP, but not phorbol esters, was able to mimic the effects of LH. Db-cAMP, forskolin and cholera toxin were able to cause down-regulation of the LH receptor by an inhibition of internalization followed by proteolysis in MA10 and MLTC-1 cells. In contrast no effect of PMA or db-cAMP was observed on the internalization of hormone in rat Leydig cells.

It has been reported that in mouse Leydig cells LH-induced desensitization and down-regulation are concurrent (Freeman and Ascoli, 1981). LH-induced desensitization was mimicked by forskolin and PMA (Lefèvre et al., 1985; Rebois et al., 1985). Previously though, no report of a loss in binding sites was reported with either PMA or forskolin. In our studies forskolin, cholera toxin and db-cAMP caused a loss in binding sites under the same conditions that cause desensitization. LH-induced desensitization in rat Leydig cells is not concurrent with down-regulation. In rat Leydig cells, PMA can cause desensitization (Dix et al., 1987), but cyclic AMP does not (Dix et al., 1982). Our studies show that no loss of binding sites occurs in rat Leydig cells with db-cAMP or with LH under conditions that cause desensitization. These results indicate that there may be differences in the sites and mechanisms of phosphorylation of the LH receptor in the mouse and rat Leydig cells.

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